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Platelet-derived nerve growth factor supports the survival of cholinergic neurons in organotypic rat brain slices

Kathrin M. Kniewallner, **Natalia Grimm**, and **Christian Humpel***
Laboratory of Psychiatry and Exp. Alzheimer Resaerch, Department of Psychiatry and Psychotherapy, Innsbruck Medical University, Anichstr. 35, A-6020 Innsbruck, Austria

Abstract

Platelets play a role in repair of vessels and contain different growth factors, including nerve growth factor (NGF). Since NGF is the most potent growth factor to support survival of cholinergic neurons, we aimed to study the effects of platelet-derived NGF on cholinergic neurons in organotypic brain slices. Brain slices of the nucleus basalis of Meynert (nBM) were cultured with or without NGF (10 ng/ml) or platelet extracts (100 μg/ml) or fresh platelets (10^8 platelets/ml). In order to enhance NGF in platelets recombinant NGF (100 ng) was loaded into platelets using ultrasound (3 h). Our data show that recombinant NGF markedly supports survival of cholinergic neurons. The addition of fresh platelets showed a tendency for enhancing cholinergic neuron numbers, while platelet extracts had no effects. Ultrasound was highly effective to load recombinant NGF into platelets. The addition of NGF-loaded platelets markedly enhanced cholinergic neuron numbers. In conclusion, our data provide evidence that NGF-derived platelets may counteract cell death of cholinergic neurons.

Keywords

Platelets; Nerve growth factor; Neuroprotection; Cholinergic neurons

1. Introduction

Platelets (thrombocytes) play a central role in the blood clotting process and repair of vessels. Platelets contain several biogenic substances in their secretory granules, which are released upon activation [1]. Platelets contain many different growth factors such as e.g. two neurotrophins: NGF and brain-derived neurotrophic factor (BDNF). The role of NGF in platelets is not well-investigated, but it has been shown that NGF can induce platelet aggregation [2]. We have recently shown that NGF is spontaneously released upon activation within minutes, and different to the release of BDNF [3]. However, the release and storage of NGF and BDNF from platelets is not well understood, and it seems likely that both are not stored in the same granules or differentially released upon stimulation, or alternatively also differentially taken up and re-stored [3]. In the brain, mature NGF is

*Corresponding author. Tel.: +43 512 504 23712; fax: +43 512 504 23713. christian.humpel@i-med.ac.at (C. Humpel).

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processed from a large precursor protein proNGF, stored in neurons and the release of NGF is highly regulated by neuronal activity [4].

The cholinergic system plays an important role in cognition and memory and is severely impaired in neurodegenerative diseases such as Alzheimer's disease. A lack of the neurotransmitter acetylcholine directly correlates with cognitive decline [5]. Nerve growth factor (NGF) is the most potent trophic substance to maintain and support the survival of cholinergic neurons [6]. Thus, the aim of the present study was to explore if platelet-derived NGF may counteract cell death of cholinergic neurons in an organotypic brain slice model. In the case that endogenous NGF is not potent enough we plan to load recombinant exogenous NGF into platelets.

2. Methods

2.1. Platelet isolation from rat

For the animal experiments adult Sprague Dawley rats were used. The rats were anaesthetized with a high dose of thiopental (Sandoz) and blood was directly drawn from the heart by using a 21 ga butterfly blood collection system (BD Valu-Set, BD). The blood was collected in ethylenediaminetetraacetate (EDTA) tubes (S-monovettes, Sarsted) and gently mixed. Platelets were isolated as described in detail [7]. Immediately after blood collection, anticoagulated rat blood was centrifuged at 250 × *g* for 15 min to obtain platelet rich plasma (PRP). All centrifugation steps were performed at room temperature. PGI_2 (Prostaglandin, 500 nM, Sigma) was added to prevent platelet activation during processing. Platelets were separated from PRP by centrifugation at 2300 × *g* for 10 min and washed in calcium-free Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose, pH 6.5). After further centrifugation at 2300 × *g* for 10 min platelets were finally resuspended in 200 μl Tyrode buffer (adjusted to pH 7.4) and further processed.

2.2. Loading of NGF into platelets and platelet rich plasma

Blood was collected from rats, centrifuged 250 × *g* 15 min and 2/3 of the upper layer (PRP) was collected. To load NGF into platelets, 400 μl of PRP or freshly isolated platelets were incubated with or without 100 ng recombinant NGF (Sigma) either on ice or in an icecold ultrasound bath (Bandelin Sonorex RK514, power 215; 860 W, 35 kHz frequency) for 3 h. After ultrasound loading of PRP, platelets were isolated as described.

2.3. FACS analysis

FACS analysis on freshly isolated platelets was performed with a BD FACs (FACScan; Becton Dickinson). Briefly 10 μl of isolated platelets (1:10 diluted) were incubated with antibodies against CD31 (Miltenyi Biotec 1:10) or CD61 (Miltenyi Biotec 1:10) or with IgG1 (Miltenyi Biotec 1:25) as a negative control in 50 μl FACs buffer (2mMEDTA, 0.5% FCS, ad 100 ml PBS, pH 7.1) for 30 min at 4 °C. In order to study apoptosis, freshly isolated platelets or platetels incubated for 3 h at 4 °C or in an ultrasound bath were centrifuged, and cells dissolved in Tyrode and 2 μl were incubated with 5 μl Annexin V-FITC (Miltenyi Biotec) in 100 μl binding buffer for 30 min at 4 °C, and then washed and analyzed by FACS.

2.4. Organotypic brain slice cultures

All experiments conformed to Austrian guidelines on the ethical use of laboratory animals, and all efforts were made to minimize their suffering and the number of animals used. The nucleus basalis of Meynert (nBM) [7,8] of postnatal day 8–10 Sprague Dawley rats (Himberg, Austria) was dissected under aseptic conditions and 400 μm slices were cut with a tissue chopper (McIlwain, Westbury, NY, USA). Six slices were placed on a 30-mm diameter Millicell-CM 0.4-μm membrane insert (Millipore, Vienna, Austria) where they became attached after two weeks of incubation. Slices were cultured in 6-well plates (Greiner, Frickenhausen, Germany) at 37 °C and 5% CO_2 with 1.2 ml/well of the following culture medium: 50% MEM/HEPES (Invitrogen, Lofer, Austria), 25% heat-inactivated horse serum (Invitrogen), 25% Hanks' solution (Invitrogen), 2 mM $NaHCO_3$ (Merck, Darmstadt, Germany), 6.5 mg/ml glucose (Merck), pH 7.2. Brain slices were incubated with or without 10 ng/ml nerve growth factor (Sigma, Vienna, Austria). Alternatively slices were incubated with platelet extracts (100 μg/ml) or fresh isolated platelets (1×10^8 ml^{-1}). The medium was changed once a week (100%) and medium was added (100 μl) once per week. In some experiments an anti-NGF antibody (Cedarlane, clone MC51) was added at a concentration of 10 μg/ml.

2.5. Platelet extracts

In order to prepare platelets extracts, the isolated cells (as pellets) were dissolved in 150 μl PBS containing a protease inhibitor cocktail (Sigma), sonicated on ice (10 s, 125 W/cm^2, 140 μm amplitude, 100%), and centrifuged (10 min, 4 °C, 14,000 × *g*). The supernatant was collected and frozen at −80 °C until use. Total protein was determined by Bradford using Coomassie Brilliant Blue G250 dye (BioRad Laboratories, Vienna, Austria).

2.6. Immunohistochemistry

Isolated platelets were spotted onto collagen-coated glass slides, air dried, and then processed for immunohistochemistry on the same day. Brain slices were fixed for 3 h at 4 °C with 4% paraformaldehyde/10 mM phosphate-buffered saline (PBS) and stored at 4 °C in PBS until use. Immunohistochemistry was performed as described previously [7]. Cells/slices were washed with 0.1% Triton/PBS for 30 min and pretreated for 20 min with 20% methanol (Roth, Karlsruhe, Germany)/1% H_2O_2 (Merck)/PBS. After rinsing, the slices were blocked for 30 min with 20% horse serum (Invitrogen)/0.2% BSA/T-PBS (Serva, Heidelberg, Germany) and incubated for two days with primary antibodies against choline acetyltransferase (ChAT, 1:750, Millipore), CD61 (Miltenyi Biotec) or NGF (Sigma). Cells/slices were washed again and incubated with secondary biotinylated anti-goat (ChAT) or anti-mouse (CD61) or anti- rabbit (NGF) (1:200, all Vector Labs, Vienna, Austria) for 1 h. Cells/slices were washed in PBS, incubated in ABC-Vectastain reagent (Vector Labs) for 30 min, washed in 50 mM tris-buffered saline (TBS) and detected by means of 0.5 mg/ml 3,3′-diaminobenzidine (DAB, Sigma) including 0.003% H_2O_2 in TBS as a substrate. The cells/slices were mounted on glass slides, air-dried and coverslipped with Entellan (Merck).

2.7. NGF ELISA

NGF levels were measured using commercial Enzyme-Linked Immuno-Sorbent Assay (ELISA) kits (Promega, Mannheim, Germany). Detection of NGF was performed as described by us recently [3]. Briefly, 100 μl standards or samples were added to coated wells and incubated for 6 h at room temperature. After washing, the detection antibodies were added and samples were incubated over night at 4 °C. Wells were washed again and incubated with horseradish peroxidase conjugate for 2.5 h. After washing, TMB one solution was added. After 10 min the reaction was stopped and the absorbance was measured at 450 nm in a Zenyth 3100 ELISA reader.

2.8. Quantification and statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) with a subsequent Fisher LSD posthoc test and by Students *t*-test; A p-value <0.05 was considered as statistically significant.

3. Results

3.1. Characterization of platelets

Platelets from adult rats were isolated and characterized by FACS. FACS analysis revealed a single population of 96 ± 1 (n = 4) % (Fig. 1A), which strongly stained for CD31 (Fig. 1D) and CD61 (Fig. 1E). Immunohistochemistry against the platelet marker CD61 showed that the cells were small (4.2 ± 0.4 μm, n = 3) and were all positive (Fig. 1H). Immunohistochemistry without primary antibody showed only background (Fig. 1G).

3.2. Cholinergic neurons in slices incubated with NGF or platelets

Immunohistochemistry against cholineacetyltransferase (ChAT) was used to stain cholinergic neurons in brain slices. Brain slices were dissected from the basal nucleus of Meynert of a postnatal day10 rat (Fig. 2A). When slices were incubated without NGF very few cholinergic neurons survived (Figs. 2B and 3). Slices incubated with 10 ng/ml recombinant NGF contained significantly more cholinergic neurons (approx. 90 neurons) in nBM brain slices (Figs. 2C and 3). When slices were incubated with recombinant NGF together with an anti-NGF antibody, the number of ChAT+ neurons markedly decreased and was not different to controls (Fig. 3).

3.3. Cholinergic neurons in slices incubated with platelets

When slices were incubated with fresh isolated platelets (1×10^8 platelets per well), the number of cholinergic neurons was only slightly (p = 0.06) enhanced compared to controls (Fig. 3). This effect was again abolished when the anti-NGF antibody was added (Fig. 3). Platelet extracts alone did not have any protective effect on cholinergic neurons (Fig. 3).

3.4. Loading NGF into platelets

Our data show that loading of recombinant NGF into PRP yields a better and more reproducible method as loading into freshly isolated platelets (Table 1). When platelets were incubated for 3 h at 4 °C the FACS population became slightly smaller (Fig. 1B), but

markedly decreased after incubation in an ultrasound bath for 3 h (Fig. 1C). No Annexin-V apoptosis was seen in platelets either incubated at 4 °C or in an ultrasound bath for 3 h (Fig. 1F). PRP treated with 100 ng NGF in an ultrasound bath revealed markedly higher platelet NGF levels (Table 1) compared to controls or PRP not treated in an ultrasound bath (Table 1). Protein levels were not affected. Immunohistochemistry against NGF revealed several positive labeled cells after ultrasound bath loading (Fig. 1I). The amount of released NGF was approx. 200 pg/ml × week for control platelets, but increased to approx. 1300 pg/ml × week for NGF-loaded platelets (Table 1). When slices were incubated with NGF-loaded platelets, the number of cholinergic neurons was significantly enhanced (Figs. 2D and 3).

4. Discussion

In the present study we show that platelet-derived NGF may support the survival of cholinergic neurons. Recombinant exogenous NGF can be loaded into platelets by ultrasound and has a more potent effect than endogenous NGF.

4.1. Organotypic brain slices

The organotypic brain slice model is well-established in our working group and serves as a validated tool to study toxic, degenerative and developmental changes as well as synaptic recovery, survival and cell death of neurons [8–10]. In this model cholinergic neurons are axotomized, however, the normal cytoarchitecture is retained similar to the in vivo situation and functional connections including transport and diffusion probabilities are maintained. The brain tissue is derived from postnatal day 10 brains and therefore it is not completely comparable to adult brains, which is a limitation of the present study. Cholinergic neurons are stained by antibodies against choline acetyltransferase, and a decline correlates to cell death of cholinergic neurons. In nBM slices cholinergic neurons rapidly degenerate when incubated without NGF, but the number of remaining neurons can be rescued by NGF application at any time point [8–10]. We have shown that NGF concentrations as low as 0.1 ng/ml are sufficient to support cholinergic neurons in brain slice cultures [11].

4.2. Characterization of platelets

Platelets are about 3-5 μm small cells and are processed from megakaryocytes and do not have a nucleus. Rise of intracellular calcium leads to platelet activation and shape change [1], resulting in exocytosis of granules and the subsequent secretion of their contents. Platelets contain three different kinds of secretory granules: (1) dense bodies storing ADP, ATP, calcium, serotonin (2) alpha granules containing BDNF, PDGF, TGF-β and platelet factor 4 and possibly NGF (3) lysosomes that contain acid hydrolases. Secretion of the granules can be induced by various stimuli, like ADP and thrombin [3]. As platelets contain many inflammatory mediators (matrix metalloproteinases, chemokines, cytokines) and growth factors, they may have an important role in neuroinflammation [1]. For platelets characterization we used FACS analysis for CD61, a specific antigen that is expressed on the surface of resting platelets. CD61 is also known as integrin β3 and is well established as a platelet marker. It is present as a subunit for the fibrinogen-receptor, playing a role in blood clotting [12]. Further, platelets also express CD31 (cluster of differentiation 31), a

member of the immunoglobulin superfamily, that is expressed on the surface of circulating platelets, monocytes, neutrophils, and particular T-cell subsets.

4.3. Platelet endogenous NGF and cholinergic neurons

NGF is a neurotrophic substance that is synthesized and released by cells in the cortex and hippocampus and its secretion in the brain is strongly regulated and underlies the influences of neuronal activity. It is produced in its immature form from proNGF, a 36 kDa protein and is transported retrogradely from the synapses to their cell bodies. [13]. Many studies have shown that brain-derived NGF enhances the survival of cholinergic neurons [10,14,15], however, the role of platelet-derived NGF is still not well investigated. We have recently shown that rat platelets spontaneously release almost all of their NGF very fast [3], which is different to the release of BDNF and it could be that NGF and BDNF are not stored in the same secretory granules. In the present study endogenous NGF was detectable in rat platelets and it could partly counteract cell death of cholinergic neurons. However, when platelet extracts were added, the number of cholinergic neurons was not affected. It is likely, that many proteases are released from extracted platelets, which may counteract the protective effect of NGF. In order to proof that the protective effect of platelets on cholinergic neurons was due to endogenous NGF, we incubated slices with an anti-NGF-antibody. In fact, the blocking antibody significantly counteracted the effect of platelet-derived NGF on the survival of cholinergic neurons in nBM slices.

4.4. Loading of exogenous NGF into platelets and effects on cholinergic neurons

In order to enhance the NGF content in platelets, we aimed to load recombinant exogenous NGF into platelets. Several methods to transfer proteins or to produce genetically modified cells have been reported: Bioporter protein transfer, viral mediated systems, electroporation or lipofection. Since platelets do not possess a nucleus, all plasmid derived methods are not useful. Thus, in order to transfer protein into platelets it may be important to make the cell membrane permeable. We tested two methods, ultrasound treatment or electroporation. Electroporation was not successful (data not shown), however we could show that ultrasound is suitable to load NGF into platelets.

Ultrasound has been successfully used to permeate membranes for different purposes and is a broadly utilized method to deliver drugs into cells [16]. In fact, ultrasound has been used to successfully deliver the disaccharide trehalose into human platelets [17]. Using two different frequencies, different radiation times and intensities, it has been shown that the lower frequency of 25 kHz (in comparison to 800 kHz) and a longer radiation time achieved better loading results, whereas intensity of ultrasound radiation does not seem to play an important role [17]. In our present study we show the first time that ultrasound (35 kHz) is suitable to load NGF into platelets and enhance NGF levels 6-fold. In order to demonstrate that NGF-loaded platelets have an effect on cholinergic neurons, we incubated brain slices of the nBM with fresh isolated and loaded platelets, and in fact, we show that NGF-derived from platelets significantly supports the survival of cholinergic neurons. This is in agreement with our previous data, where low levels of 0.1 ng/ml recombinant NGF support survival of cholinergic neurons in slice cultures [11]. Thus, we conclude that platelets may provide a potent source of protective NGF and could be useful as vehicles into the brain.

4.5. Perspectives

Several requirements that must be necessarily fulfilled for clinical use of NGF [18]. First, NGF must efficiently stimulate cholinergic neurons and it must be possible to deliver NGF in adequate amounts. Second, the application of NGF must be narrowed to the target region, to avoid unwanted effects [18]. Further, it would be necessary to deliver NGF continuously over years and a therapy should start as early as possible to protect still intact neurons from degeneration to come. Yet, NGF could represent a useful therapy and, moreover, NGF and anti-amyloidogenic approaches could have a synergistic effect [18,19]. However, NGF is a large molecule and will not cross the blood–brain barrier and novel delivery strategies need to be developed.

Under healthy conditions, blood cells cannot pass the BBB. However, vascular damage is a pronounced pathological event in AD and it seems likely, that blood cells can migrate to lesion sites. Especially monocytes have been shown to enter the AD brain and migrate to beta-amyloid plaques, where they may differentiate into microglia or macrophages and phagocytose aggregated amyloid [9,20,21]. In the case of platelets, a migration into the brain has not been shown. However, considering the dense vascular network in the brain and that nearly every neuron is approx. 30–50 μm apart of a vessel, it seems likely that platelets may migrate deep into the brain and release protective factors. It needs to be proven that NGF-loaded platelets may migrate into the brain and release their NGF content close to cholinergic neurons.

In conclusion, our data show that platelets contain endogenous NGF, which partly counteracts cell death of cholinergic neurons. However, platelets can be easily loaded with exogenous NGF and provide a more potent source of protective NGF.

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HIGLIGHTS

- Platelets contain endogenous nerve growth factor (NGF).
- Platelets can be loaded with recombinant NGF using ultrasound.
- Platelet-derived NGF supports survival of cholinergic neurons in brain slices.

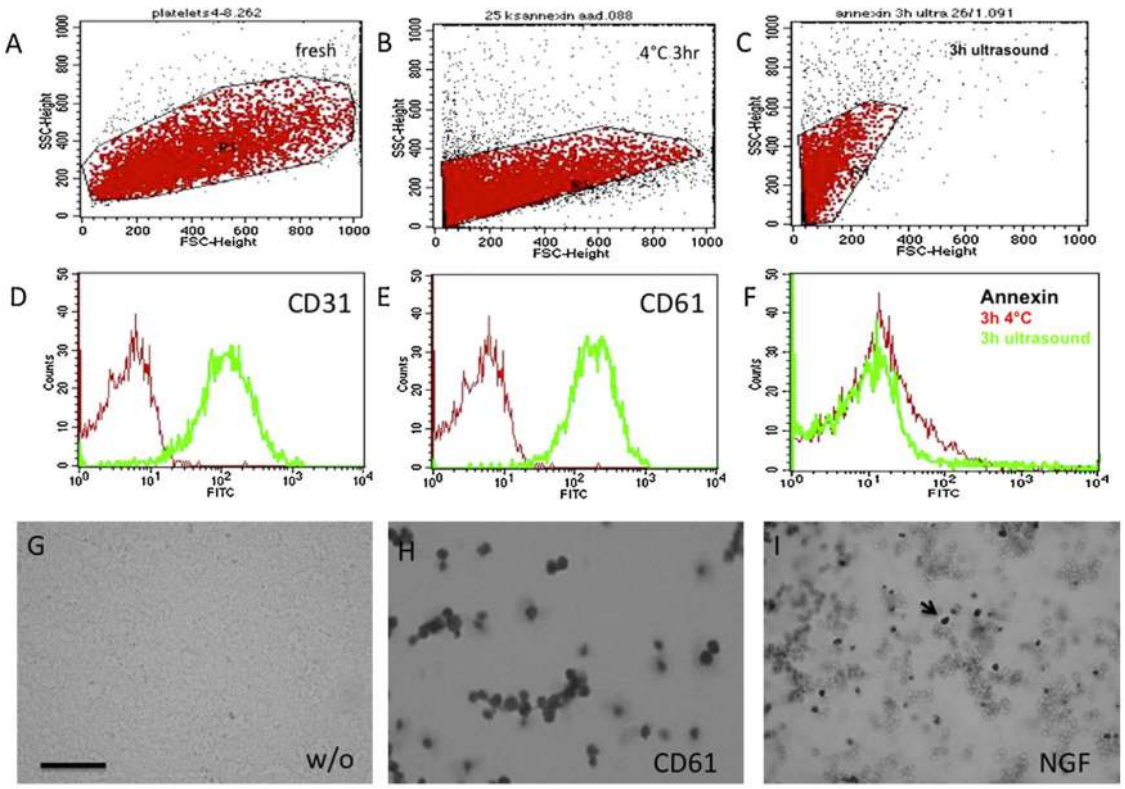

Fig. 1.

Characterization of rat platelets. FACS analysis revealed a large single cell population of freshly isolated platelets (A). This population was slightly different in platelets incubated for 3 h at 4 °C (B), and was clearly decreased in platelets incubated for 3 h in an ultrasound bath (C). Freshly isolated platelets stained positive for CD31 ((D) green) and CD61 ((E) green) compared to an IgG control ((D) and (E) red). Platelets incubated for 3 h at 4 °C ((F) green) or in an ultrasound bath ((F) red) did not show any Annexin-V positive staining ((F) green). Immunohistochemistry shows that the platelets were positive for CD61 (H), while a control without primary antibody was negative (G). When platelets were loaded (3 h ultrasound bath) with recombinant nerve growth factor (NGF), NGF-like immunoreactivity was clearly detectable in some cells ((I) arrow). Scale bar in (G) equals 15 μm (G–I). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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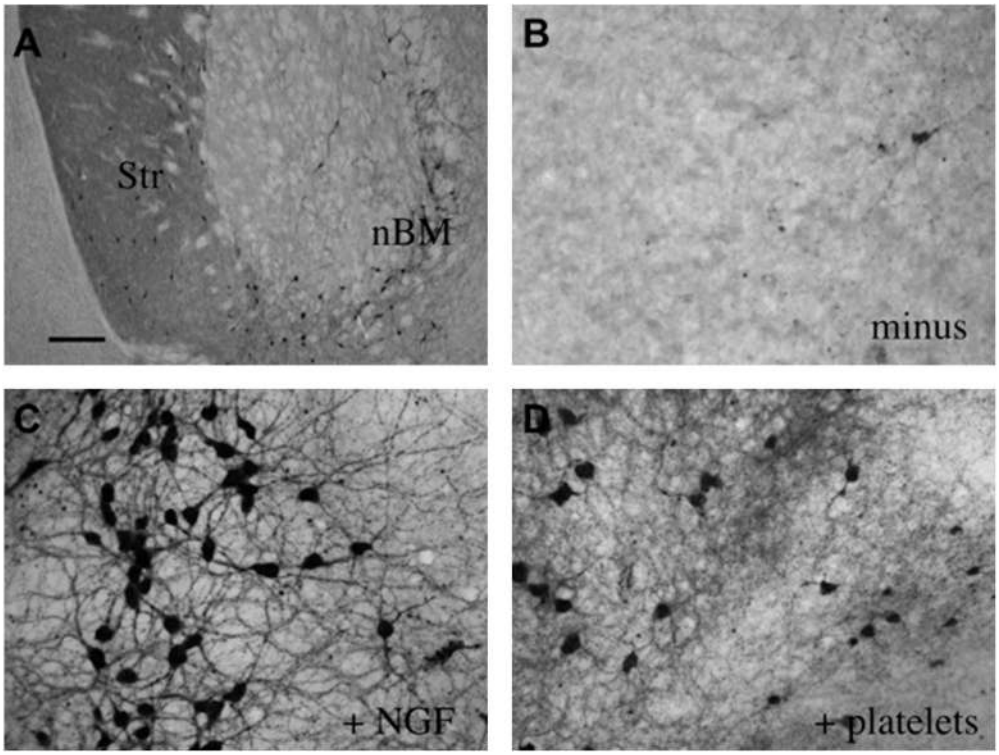

Fig. 2.

Cholinergic neurons of the nucleus basalis of Meynert (nBM) stained for cholineacetyltransferase (ChAT). Brain slices of the nBM were dissected from postnatal day 10 rats (A), incubated without (B) or with 10 ng/ml nerve growth factor (NGF, (C)), or with fresh NGF-loaded isolated platelets (1×10^8 ml^{-1}) and incubated for 2 weeks. Note that many cholinergic neurons survived when incubated with NGF. The neurons incubated with platelets were markedly higher compared to slices incubated without NGF but did not look as healthy as slices incubated with recombinant NGF. Striatum (Str) Scale bar in (A) equals 360 μm (A), 75 μm ((B)–(D)).

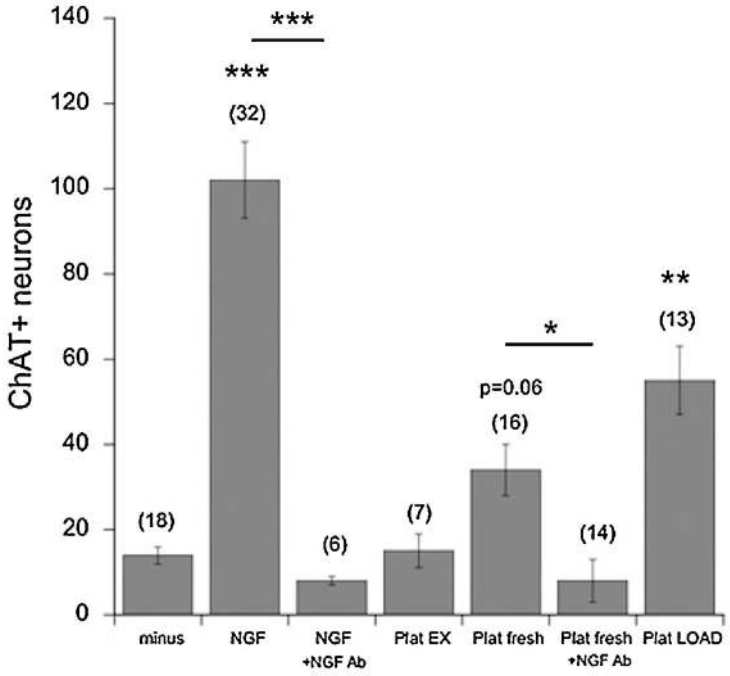

Fig. 3.

Quantitative analysis of cholinergic neurons. Slices of the nucleus basalis of Meynert were incubated with nerve growth factor (NGF) or without (minus) or fresh platelets (Plat) (1×10^8 ml^{-1}) or platelet extracts (100 μg/ml) or NGF loaded platelets (LOAD). In some experiments an anti-NGF antibody (NGF Ab) was added (10 μg/ml). Values are expressed as mean ± SEM choline acetyltransferase (ChAT+ neurons); values in parenthesis give the number of analyzed slices. Statistical analysis was performed by one way ANOVA with a subsequent Fisher LSD posthoc test (* $p < 0.05$;** $p < 0.05$; *** $p < 0.001$).

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Table 1

Loading of recombinant NGF into platelets (A) or platelet rich plasma (B).

(A) Platelets	Protein [µg/ml]	NGF [pg/mg]	NGF release [pg/ml × week × 1 × 10^8]
Minus	381 ± 60 (3)	667 ± 271 (3)	N.A.
+NGF	402 ± 117(3) ns	3768 ± 2228 (3) ns	N.A.
+NGF/USB	148 ± 73 (3) ns	5082 ± 3204 (3) ns	N.A.
(B) PRP			
Minus	354 ± 57 (17)	967 ± 255 (10)	219 ± 133 (8)
+NGF	449 ± 147 (10) ns	3152 ± 722 (9) ns	N.A.
+NGF/USB	386 ± 136 (11) ns	6455 ± 1565 (11) ***	1351 ± 460 (12)
		$p < 0.05$ vs. PRP/NGF	

Platelets (3×10^8) or platelet-rich plasma (PRP) was collected and incubated with or without 100 ng recombinant nerve growth factor (NGF) on ice or in an ultrasound bath (USB) for 3 h. Then platelets were isolated, and extracted and the total protein and the NGF content was determined. For the release experiment 1×10^8 platelets were incubated for 1 week in slice medium, and then the release of NGF was measured. Values are given as mean ± SEM, values in parenthesis give the number of single experiments. Statistical analysis was performed by one way ANOVA with a subsequent LSD posthoc test

*** ($p < 0.001$). ns, not significant; N.A., not analyzed.