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Original Paper

Role of Na^+/Ca^{2+} Exchangers in Therapy Resistance of Medulloblastoma Cells

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Key Words
NCX3 • NCKX2 • NCKX5 • Ionizing radiation • Apoptosis

Abstract
Background/Aims: Alterations of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$) are decisive in the regulation of tumor cell proliferation, migration and survival. Transport processes participating in the regulation of $[Ca^{2+}]_i$ include Ca^{2+} extrusion through K^+-independent (NCX) and/or K^+-dependent (NCKX) Na^+/Ca^{2+}-exchangers. The present study thus explored whether medulloblastoma cells express Na^+/Ca^{2+}-exchangers, whether expression differs between therapy sensitive D283 and therapy resistant UW228-3 medulloblastoma cells, and whether Na^+/Ca^{2+}-exchangers participate in the regulation of cell survival. ***Methods:*** In therapy sensitive D283 and therapy resistant UW228-3 medulloblastoma cells transcript levels were estimated by RT-PCR, protein abundance by Western blotting, cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$) from Fura-2-fluorescence, Na^+/Ca^{2+}-exchanger activity from the increase of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) and from whole cell current (I_{ca}) following abrupt replacement of Na^+ containing (130 mM) and Ca^{2+} free by Na^+ free and Ca^{2+} containing (2 mM) extracellular perfusate as well as cell death from PI -staining and annexin-V binding in flow cytometry. ***Results:*** The transcript levels of NCX3, NCKX2, and NCKX5, protein abundance of NCX3, slope and peak of $\Delta[Ca^{2+}]_i$ as well as I_{ca} were significantly lower in therapy sensitive D283 than in therapy resistant UW228-3 medulloblastoma cells. The Na^+/Ca^{2+}-exchanger inhibitor KB-R7943 (10 µM) significantly blunted $\Delta[Ca^{2+}]_i$, and augmented the ionizing radiation-induced apoptosis but did not significantly modify clonogenicity of medulloblastoma cells. Apoptosis was further enhanced by NCX3 silencing. ***Conclusions:*** Na^+/Ca^{2+}-exchanger activity significantly counteracts apoptosis but does not significantly affect clonogenicity after radiation of medulloblastoma cells.

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Introduction

Cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) is a powerful regulator of a wide variety of cellular functions, such as cell proliferation, migration, excitation, exocytosis, and cell death. Determinants of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) include Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers, which terminate increases of $[Ca^{2+}]_i$ [1-9]. Na^+/Ca^{2+} exchangers accomplish a rapid decline of $[Ca^{2+}]_i$ as the turnover rate of Na^+/Ca^{2+} exchangers by far exceeds the turnover rates of Ca^{2+} ATPases [10]. The family of Na^+/Ca^{2+} exchangers includes 6 K^+-dependent (NCKX) and 3 K^+-independent (NCX) Na^+/Ca^{2+} exchanger isoforms [11-13]. The NCX isoforms exchange three Na^+ ions for one Ca^{2+} ion, whereas the NCKX isoforms exchange one K^+ ion and one Ca^{2+} ion for four Na^+ ions [14]. The transport direction is dependent on the cell membrane potential, the Na^+ gradient, the Ca^{2+} gradient and, for the NCKX isoforms the K^+ gradient [15].

The present study explored whether medulloblastoma cells express Na^+/Ca^{2+} exchangers, whether Na^+/Ca^{2+} exchanger expression and function differs between therapy resistant UW228-3 medulloblastoma cells and therapy sensitive D283 medulloblastoma cells, and whether pharmacological inhibition of Na^+/Ca^{2+} exchangers modifies apoptosis and sensitivity of the cells after exposure to ionizing radiation.

Materials and Methods

Ethics Statement

Investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

Cell culture, transfection and irradiation

Experiments were performed in therapy sensitive D283 and therapy resistant UW228-3 medulloblastoma cells [16]. The cells were cultured in Dulbecco's RPMI media, containing 10% fetal calf serum and 1% Penicillin/ Streptomycin (Gibco ThermoFischer Scientific) solution. For gene silencing, medulloblastoma cells were transiently transfected with 50 nM NCX3 siGENOME SMARTpool or siCONTROL nontargeting siRNA pool (Dharmacon, Lafayette, CO). Transfection was performed using X-tremeGENE™ HP DNA Transfection Reagent (Roche Diagnostic). All experiments were thus conducted 24 hour after transfection. Where indicated, the cells were treated with the NCX inhibitor [17] KB-R7943 (10 µM) (Sigma, Taufkirchen, Germany). Irradiation was performed at 37°C using a Gulmay RS225 X-ray machine with a dose rate of 1.0 Gy/minute and the exposure factors of 200 kVp, 15 mA and 0.5-mm Copper additional filtering.

Real-time PCR

Total RNA was extracted from medulloblastoma cells in TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After DNAse digestion, reverse transcription of total RNA was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany). Real-time polymerase chain reaction (qRT-PCR) of the respective genes were set up in a total volume of 15 µl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, Hilden, Germany) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec., 58°C for 15 sec and 68°C for 20 sec. For amplification the following primers were used (5`->3`orientation):

for NCX1:
fw: acaagaggtatcgagctggc
rev: atgccatttctcgcctagc
for NCX2:
fw: cgctggtgttcaaaccag
rev: gacgaccacgcaggcat
for NCX3:
fw: gcattgccagggtcattgtct

rev: ccataagggtcaggttggaga
for NCKX1:
fw: tccacgcagaagatggtg
rev: gtgatggaggggatagcg
for NCKX2:
fw: gagacagatacacagagcacagg
rev: gagaatagtacagatcacgccc
for NCKX3:
fw: atgacagcagcaactgcga
rev: tcattgatcaacatgcgactgg
for NCKX4:
fw: tcaaagttcgcaggaggcg
rev: attcactggggccatcaact
for NCKX5:
fw: ctccatcatcggagtttcc
rev: cttcctaccctccctggaa
for NCKX6:
fw: cgtgctggttaccacagtgg
rev: cttccgtggcagggtcag
for GAPDH:
fw: tgagtacgtcgtggagtccac
rev: gtgctaagcagttggtggtg

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad) and all experiments were done in duplicate. The house-keeping gene GAPDH (glycerinaldehyde-3-phosphate-dehydrogenase) was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described earlier [18, 19].

Ca^{2+} measurements

Fura-2 fluorescence was utilized to determine intracellular Ca^{2+} activity [20]. Cells were loaded with Fura-2/AM (2 μM, Invitrogen, Goettingen, Germany) and 1 μM thapsigargin (Sigma, St. Louis, MO, USA) for 20-60 min at 37°C. Cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40×/1.30 oil) built in a fluorescence microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA).

Na^+/Ca^{2+} exchanger activity was estimated from the changes in cytosolic Ca^{2+} activity upon replacement of extracellular Na^+ by N-methyl-d-glucamine (NMDG). The standard Na^+ containing solution was composed of (in mM): 130 NaCl, 0 KCl, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4 and the Na^+-free solution of (in mM): 90 NMDG, 0 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4. For determination of NCKX activity the Na^+ containing solution was composed of (in mM): 130 NaCl, 40 KCl, 20 TAE^+, 2 $MgSO_4$, 10 HEPES, 5 glucose, pH 7.4 and the Na^+-free solution of (in mM): 90 NMDG, 40 KCl, 20 TAE^+, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 glucose, pH 7.4. For quantification of Ca^{2+} entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following removal of Na^+.

Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode according to Hamill et al. [21]. The cells were continuously superfused through a flow system inserted into the dish. Borosilicate glass pipettes (2-5 MΩ tip resistance; Harvard Apparatus, Kent, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered. The offset potentials between both electrodes were zeroed before sealing. Whole-cell currents elicited by changing the bath solutions were measured during a continuous 40 second square wave voltage pulse to -80 mV. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The outward currents, defined as flow of positive charge from the cytoplasmic to

the extracellular membrane face, are positive currents and depicted as upward deflections of the original current traces. To measure Na^+/Ca^{2+} exchanger-mediated currents, a Na^+-based pipette solution was used (in mM): 120 NaCl, 40 KCl, 20 TEA-Cl, 2 $MgCl_2$, 2 Mg-ATP, 10 HEPES (pH 7.2/CsOH) and 1 µM free Ca^{2+}. The external first solution contained (in mM): 130 NaCl, 20 TEA-Cl, 2 $MgCl_2$, 5 glucose, 10 HEPES, 0.5 EGTA (pH 7.5/CsOH). Na^+/Ca^{2+} exchange currents were elicited by switching from the first bath solution to a bath solution that contained (in mM): 130 NMDG-Cl, 20 TEA-Cl, 2 $MgCl_2$, 2 $CaCl_2$, 5 glucose and 10 HEPES (pH 7.5/CsOH). The KCl content of the bath solutions was either 0 or 40 mM.

Clonogenic assay

Post-irradiation cell survival was analyzed by means of colony formation assay, as described earlier [22]. In brief, cells were seeded in 100 mm culture dishes (1000 cells/dish) and 24 h later were treated with DMSO or KB-R7943 (10 µM). The final concentration of DMSO in all conditions was adjusted to be similar (0.1%). At 24 h post-treatment, cultures were mock irradiated or irradiated with 2 Gy, 4 Gy, or 6 Gy (X-rays, 200 kVp, 15 mA, 0.5 mm Cu additional filtering) at 37 °C. Thereafter, cultures were incubated for ten days to allow colony growth. The culture dishes were stained with 0.05% w/v crystal violet solution, colonies with 50 cells or more were counted and the clonogenic fraction of irradiated cells was calculated based on the plating efficiency of non-irradiated cells.

Determination of apoptosis

To determine apoptosis, after indicated treatments 10^5 cells/100 µl in complete RPMI were centrifuged at 1000 rpm for 5 minutes at 24°C, added to 200 µl of PBS containing 50 µg/ml propidium iodide (Mabtag, Germany) and stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) and incubated at room temperature in the dark for 20 minutes. The cells were washed once at 1000 rpm for 5 minutes, resuspended in PBS and measured immediately with an excitation wavelength of 488 nm and an emission wavelength of 585 nm (FL-2) with flow cytometry [23] utilizing a FACS Calibur (BD, Heidelberg, Germany).

Statistical analysis

Data are provided as means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test, one sample *t*-test or ANOVA (Dunnett's test) where applicable. Results with $p<0.05$ were considered statistically significant.

Results

The present study explored whether Na^+/Ca^{2+} exchangers play a role in migration and survival of medulloblastoma cells. Quantitative real-time-PCR (qRT-PCR) was employed to quantify the transcript levels of Na^+/Ca^{2+} exchanger isoforms in therapy sensitive D283 and therapy resistant UW228-3 medulloblastoma cells. As illustrated in Fig.1, both cell lines express the Na^+/Ca^{2+} exchanger isoforms NCX3, NCKX2 and NCKX5. The transcript levels of all three isoforms were significantly higher in therapy resistant UW228-3 than in therapy sensitive D283 medulloblastoma cells (Fig. 1). As shown for NCX3, the carrier protein abundance was again significantly higher in therapy resistant UW228-3 than in therapy sensitive D283 medulloblastoma cells (Fig. 2).

In order to estimate cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), medulloblastoma cells were loaded with Fura-2/ AM and Fura-2 fluorescence taken as a measure of $[Ca^{2+}]_i$. Na^+/Ca^{2+}-exchanger activity was derived from the increase of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) after the abrupt replacement of a Na^+-containing (Na^+ 130 mM) and Ca^{2+}-free extracellular perfusate by a Na^+-free and Ca^{2+}-containing (2 mM) extracellular solution. Prior to Na^+ removal, the Fura2-fluorescence ratio was similar in therapy sensitive D283 (0.65 ± 0.01 a.u., n = 5) and therapy resistant UW228-3 (0.71 ± 0.01 a.u., n = 5) medulloblastoma cells. As illustrated in Fig. 3, the removal of external Na^+ and addition of external Ca^{2+} was followed by an increase of $[Ca^{2+}]_i$, which was significantly more pronounced in therapy resistant UW228-3 than in therapy sensitive D283 medulloblastoma cells.

Na^+/Ca^{2+}-exchanger activity was further estimated from whole cell currents measured utilizing the patch clamp technique. The current was recorded following abrupt replacement

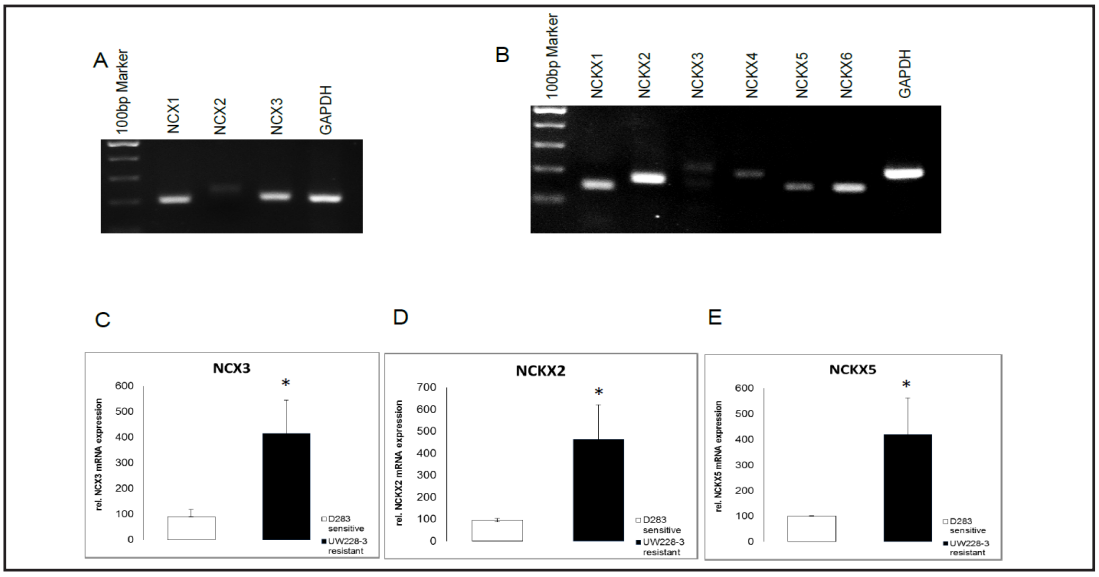
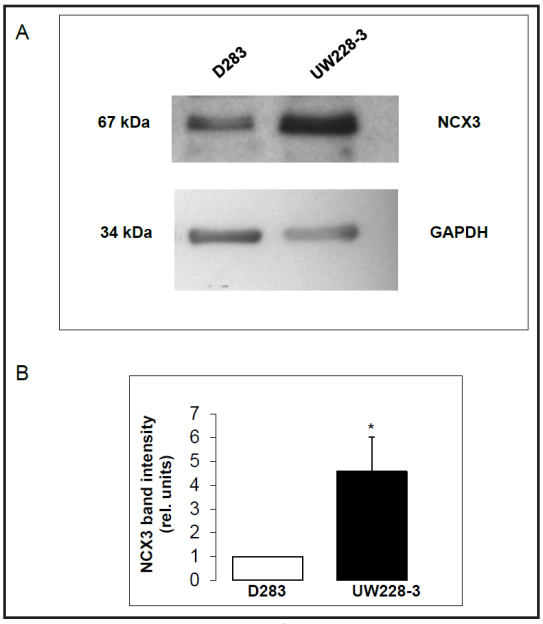


Fig. 1. Transcript levels of Na^+/Ca^{2+} exchanger isoforms NCX3, NCKX2 and NCKX5 in therapy sensitive and therapy resistant medulloblastoma cells. A,B. Original RT-PCR of NCX (A) and NCKX (B) isoform transcripts in therapy sensitive D283 medulloblastoma cells. C-E. Arithmetic means (± SEM, n = 9 preparations) of NCX3 (C), NCKX2 (D), and NCKX5 (E) over GAPDH transcript levels in therapy sensitive D283 (white bars) and therapy resistant UW228-3 (black bars) medulloblastoma cells. *($p<0.05$) indicates statistically significant difference from therapy sensitive medulloblastoma cells (student´s t-test).

Fig. 2. Protein level of Na^+/Ca^{2+} exchanger isoform NCX3 in therapy sensitive and therapy resistant medulloblastoma cells. A. Original Western blot of NCX3 and GAPDH in therapy sensitive D283 (left) and therapy resistant UW228-3 (right) medulloblastoma cells. B. Arithmetic means (± SEM, n = 4 preparations) of NCX3 over GAPDH protein levels in therapy sensitive D283 (white bars) and therapy resistant UW228-3 (black bars) medulloblastoma cells. *($p<0.05$) indicates statistically significant difference from therapy sensitive medulloblastoma cells (student´s t-test).



of Na^+-containing /Ca^{2+}-free extracellular perfusate by Na^+-free/ Ca^{2+}-containing (2 mM) solution. The removal of external Na^+ and addition of Ca^{2+} was followed by an outward current, which was significantly larger in therapy resistant UW228-3 than in therapy sensitive D283 medulloblastoma cells (Fig. 4).

Additional experiments were performed in the absence and presence of the Na^+/Ca^{2+} exchanger inhibitor KB-R7943. As illustrated in Fig. 5, the increase of cytosolic Ca^{2+} activity following removal of external Na^+ and addition of external Ca^{2+} was significantly blunted by the Na^+/Ca^{2+} exchanger inhibitor KB-R7943 (10 µM).

A further series of experiments addressed the impact of the Na^+/Ca^{2+} exchangers on the therapy resistance of medulloblastoma cells. To this end, therapy resistant and therapy

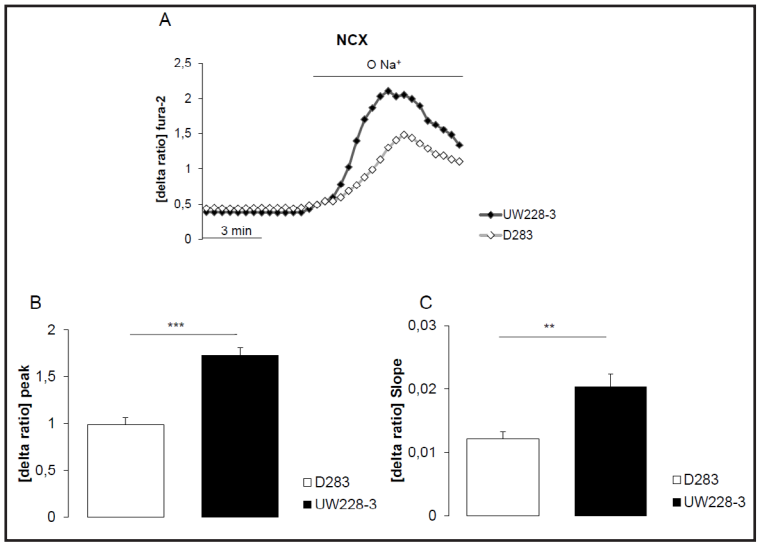


Fig. 3. NCX mediated Ca^{2+} entry in therapy sensitive and therapy resistant medulloblastoma cells. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded therapy sensitive D283 (open diamond) and therapy resistant UW228-3 (closed diamond) medulloblastoma cells prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+}. B,C. Arithmetic means (± SEM, n = 5; 73 - 67 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in therapy sensitive D283 (white bars) and therapy resistant UW228-3 (black bars) medulloblastoma cells prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+}. ** ($p<0.01$), *** ($p<0.001$) indicate statistically significant difference from therapy sensitive medulloblastoma cells (ANOVA).

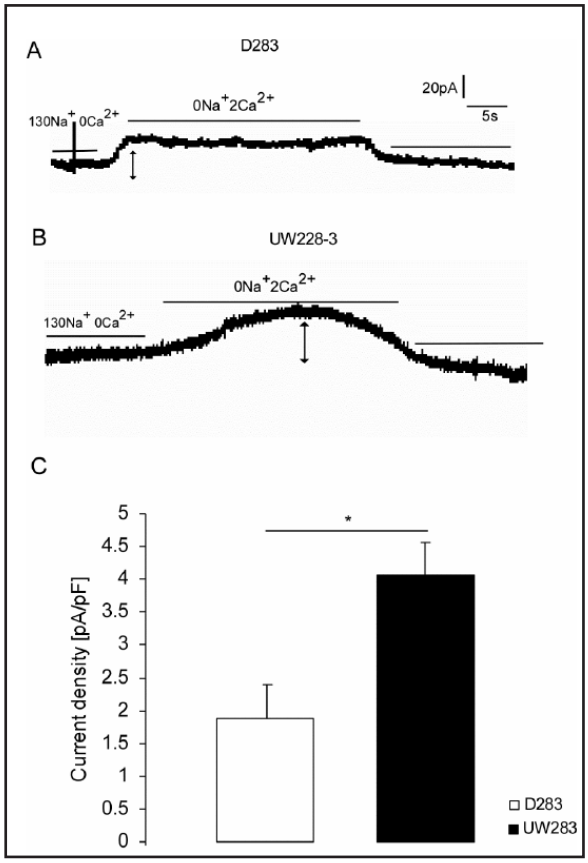


Fig. 4. Na^+/Ca^{2+} exchanger currents in therapy sensitive and therapy resistant medulloblastoma cells. A,B. Original tracings of whole cell currents in therapy sensitive D283 (A) and therapy resistant UW228-3 (B) medulloblastoma cells recorded at -80 mV during the switch between external solutions that contained either 130 mM Na^+ and no Ca^{2+} or 2 mM Ca^{2+} and no Na^+. The internal solution stimulated Na^+ - and Ca^{2+} overload (1 µM free Ca^{2+}, 120 mM Na^+). Cesium and TEA^+ were present in the solutions to block K^+ channel currents. C. Arithmetic means (± SEM, n = 5 - 7 cells) current density changes (ΔI, pA/pF) at -80 mV in therapy sensitive D283 (white bars) and therapy resistant UW228-3 (black bars) medulloblastoma cells induced by the switch between external solutions containing (in mM) 130 Na^+, 0 Ca^{2+} or 0 Na^+, 2 Ca^{2+}. * ($p<0.05$) indicates statistically significant difference from therapy sensitive medulloblastoma cells (student´s t-test).

sensitive medulloblastoma cells were irradiated with 6 Gy in the absence or presence of the Na^+/Ca^{2+} exchanger inhibitor KB-R7943 (10 µM). As a result, exposure to ionizing radiation significantly increased the percentage of propidium iodide containing and annexin-V binding medulloblastoma cells, an effect significantly more pronounced in therapy sensitive D283 than in therapy resistant UW228-3 medulloblastoma cells (Fig. 6). The additional treatment with KB-R7943 significantly increased cell death of both, therapy sensitive D283 and therapy

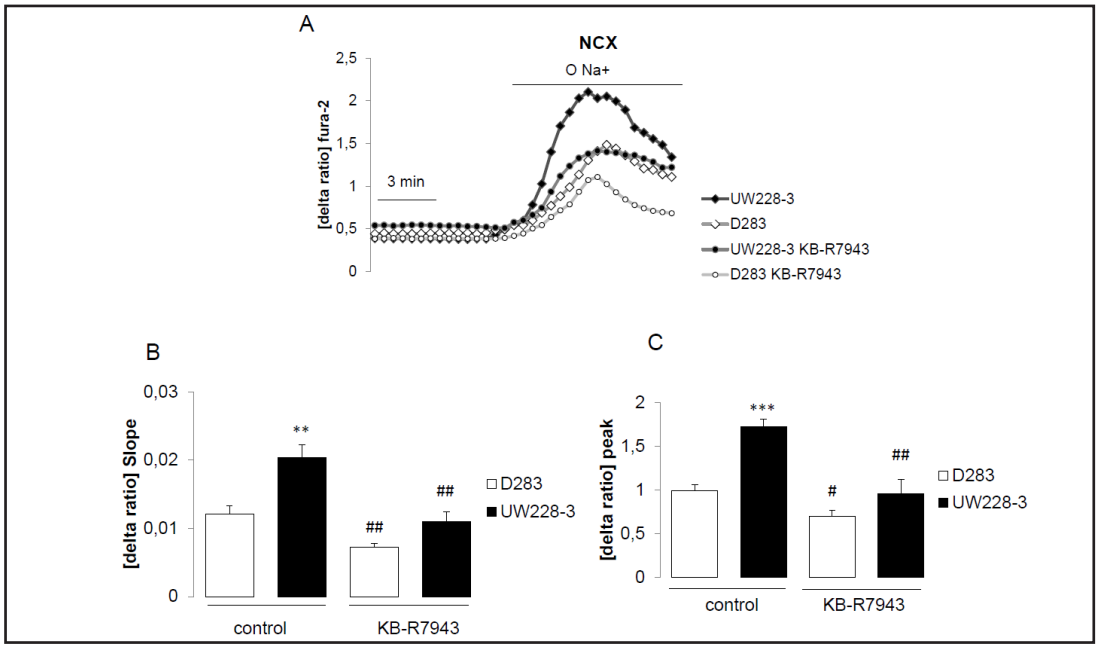


Fig. 5. Sensitivity of NCX mediated Ca^{2+} entry in therapy sensitive D283 and therapy resistant UW228-3 medulloblastoma cells to NCX inhibitor KB-R7943. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded therapy sensitive (open symbols) and therapy resistant (closed symbols) medulloblastoma cells in absence (diamonds) or presence (circles) of the NCX inhibitor KB-R7943 (10 µM) prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+}. B,C. Arithmetic means (± SEM, n = 7; 129 - 135 cells) of the peak (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in therapy sensitive (white bars) and therapy resistant (black bars) medulloblastoma cells prior to and following removal of external Na^+ (0 Na^+) and adding 2 mM Ca^{2+} in the absence (left bars, - KB-R7943) and presence (right bars, + KB-R7943) of the NCX inhibitor KB-R7943 (10 µM). ** ($p<0.01$), *** ($p<0.001$) indicate statistically significant difference from therapy sensitive medulloblastoma cells; # ($p<0.05$), ## ($p<0.01$) indicates statistical significant difference from absence of KB-R7943 (ANOVA).

resistant UW228-3 medulloblastoma cells and virtually abrogated the difference between the cell lines (Fig. 6).

As illustrated in Fig.7, silencing of NCX3 significantly increased the percentage of annexin-V-binding D283 and UW228-3 cells and significantly increased propidium iodide staining in UW228.3 cells. Thus, the NCX3 Na^+/Ca^{2+} exchanger isoform apparently protects against apoptosis of medulloblastoma cells.Treatment of D283 cells but not of UW228-3 cells with KB-R7943 is followed by a significant increase of annexin-V-binding, an observation pointing to the involvement of other KB-R7943 sensitive Na^+/Ca^{2+} exchanger isoforms or further KB-R7943 sensitive functions.

In order to further define the role of KB-R7943 sensitive Na^+/Ca^{2+} exchanger activity on radiosensitivity, a clonogenic assay was performed. As illustrated in Fig. 8, the results confirmed that radiosensitivity was higher in D283 cells than in UW228-3 cells. However, no significant difference in clonogenicity was observed between presence and absence of KB-R7943.

Discussion

The present study reveals the expression of several Na^+/Ca^{2+} exchanger isoforms in medulloblastoma cells. In both, therapy resistant and therapy sensitive medulloblastoma cells, transcript levels were detected encoding the Na^+/Ca^{2+} exchangers NCX3, NCKX2, and

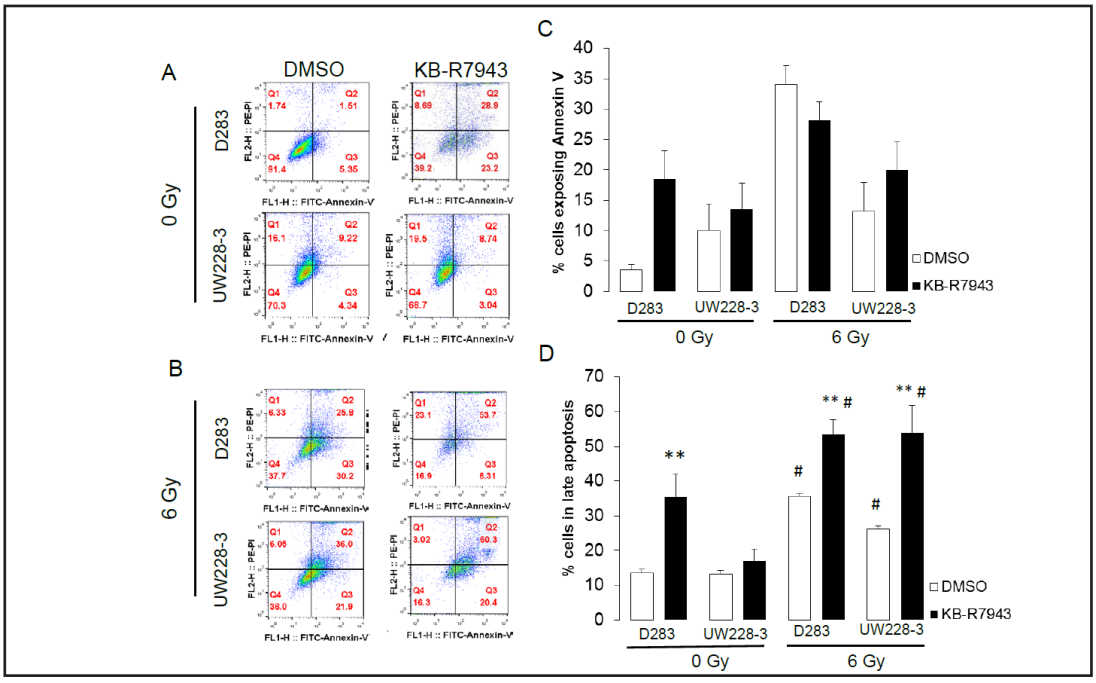


Fig. 6. Effect of NCX inhibitor KB-R7943 on radiation-induced cell death of therapy sensitive and therapy resistant medulloblastoma cells. A,B. Original blots of propidium iodide abundance versus annexin-V-binding in therapy sensitive (upper panels) and therapy resistant (lower panels) medulloblastoma cells in the absence (left panels) and presence (right panels) of the NCX inhibitor KB-R7943 (10 μM) without (A) and with (B) prior to irradiation (6 Gy). C,D. Arithmetic means (± SEM, n = 8 - 13) of the percentage of therapy sensitive D283 (white bars) and therapy resistant UW228-3 (black bars) medulloblastoma cells containing annexin-V-binding (C) and propodium iodide (D) in the absence (left bars, - KB-R7943) and presence (right bars, + KB-R7943) of NCX inhibitor KB-R7943 (10 μM) without and with prior irradiation (6 Gy). ** ($p<0.01$), indicate statistically significant difference from absence of the NCX inhibitor KB-R7943; ## ($p<0.01$) indicates statistical significant difference from absence of irradiation (6 Gy); (ANOVA).

NCKX5. The expression of NCX3 is further shown at the protein level. The transcript levels of NCX3, NCKX2, and NCKX5, protein abundance of NCX3, increase of $[Ca^{2+}]_i$ following reversal of Na^+/Ca^{2+} exchange, as well as Na^+/Ca^{2+} exchanger current were significantly lower in therapy sensitive D283 than in therapy resistant UW228-3 medulloblastoma cells. The Na^+/Ca^{2+}-exchanger inhibitor KB-R7943 (10 μM) significantly blunted $\Delta[Ca^{2+}]_i$, and significantly augmented the radiation-induced cell death of medulloblastoma cells. NCX3 silencing similarly enhanced apoptosis, an observation underscoring a role of NCX3 in cell survival. However, KB-R7943 significantly augmented the annexin-V-binding of therapy sensitive D283 cells with silenced NCX3, an observation pointing to NCX3 independent stimulation of cell membrane scrambling by the inhibitor. The clonogenic assay confirmed the relative therapy resistance of UW228-3 cells but did not confirm the impact of KB-R7943 on tumor cell survival. At least in solid tumors, radiation-induced apoptosis is only marginally involved in radiation toxicity and clonogenic activity [24, 25]. Thus, the impact of Na^+/Ca^{2+} exchanger on tumor cell survival following radiation remains uncertain.

According to previous observations in ovary carcinoma cells, therapy sensitivity is decreased by enhanced expression of the Ca^{2+} release activated channel protein Orai1 [26]. The Orai isoforms Orai1, 2, or 3 [27-30] and their regulators STIM 1 or 2 [31-33] are well known regulators of survival, proliferation, and migration of tumor cells [34-47].

The present study raises the possibility that Na^+/Ca^{2+} exchangers similarly impact on survival of medulloblastoma cells. Ca^{2+} oscillations require both, increase of cytosolic Ca^{2+}

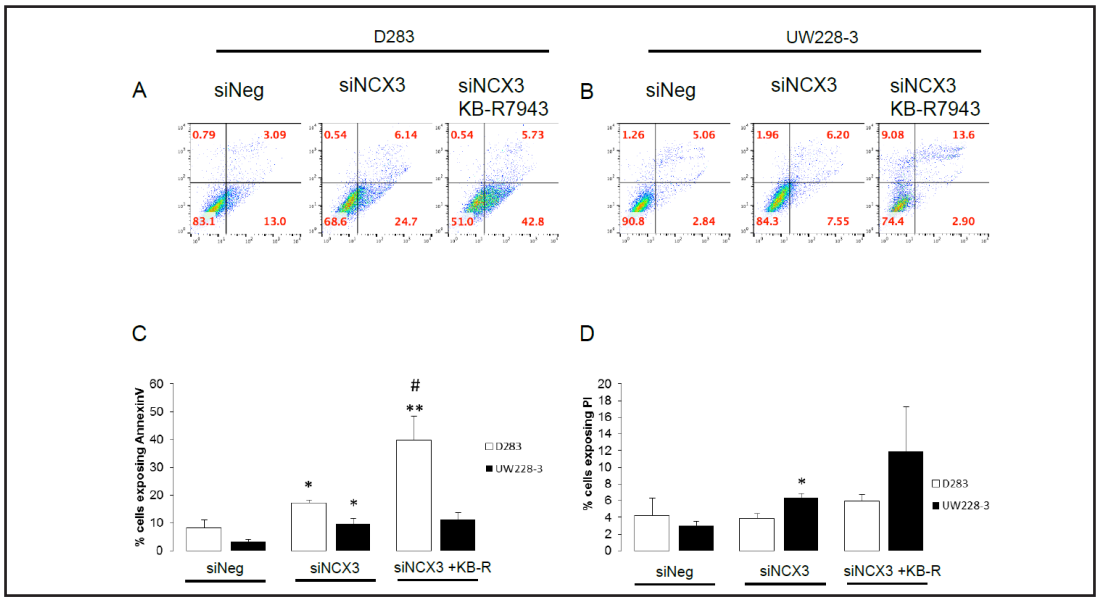
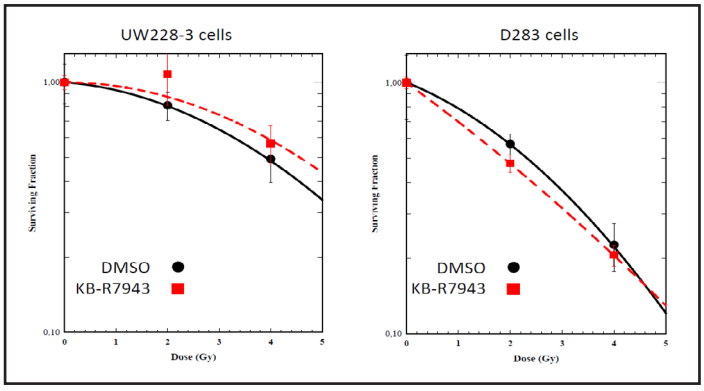


Fig. 7. Effect of NCX3 silencing on radiation-induced cell death of therapy sensitive and therapy resistant medulloblastoma cells. A,B. Original blots of propidium iodide abundance versus annexin-V-binding in (A) therapy sensitive and (B) therapy resistant medulloblastoma cells without (siNeg) and with (siNCX3) silencing of NCX3 without or with additional exposure to NCX inhibitor KB-R7943 (10 µM). C,D. Arithmetic means (± SEM, n = 4-5) of the percentage of therapy sensitive D283 (white bars) and therapy resistant UW228-3 (black bars) medulloblastoma cells containing annexin-V-binding (C) and propodium iodide (D) without (siNeg) and with (siNCX3) silencing of NCX3 without or with additional exposure to NCX inhibitor KB-R7943 (10 µM). * ($p<0.01$), **($p<0.01$) indicate statistically significant difference from absence of NCX3 silencing; # ($p<0.05$) indicates statistical significant difference from absence of KB-R7943 (ANOVA).

Fig. 8. Clonogenicity of therapy sensitive and therapy resistant medulloblastoma cells following radiation in presence and absence of KB-R7943. Arithmetic means (± SEM, n = 3) of the surviving fraction of (A) UW228-3 cells and (B) D283 cells as a function of radiation dosage in the absence (black) and presence (red) of KB-R7943 (10 µM).



activity ($[Ca^{2+}]_i$) by Ca^{2+} release and store operated Ca^{2+} entry as well as Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers [48]. The Ca^{2+} oscillations are critically important in the regulation of several cellular functions [7, 49-52], such as entrance into the S and the M phase of the cell cycle [53, 54] and support of tumor cell survival [55, 56]. In the absence of Ca^{2+} extrusion Ca^{2+} entry leads to sustained increase of $[Ca^{2+}]_i$ with subsequent triggering of apoptosis [50, 52, 57-65].

In conclusion, medulloblastoma cells express NCX3, NCKX2 and NCKX5 Na^+/Ca^{2+} exchanger isoforms. The expression of those isoforms and Na^+/Ca^{2+} exchanger activity is higher in therapy resistant UW228-3 than in therapy sensitive D228 medulloblastoma cells. Pharmacological inhibition of Na^+/Ca^{2+} exchange and NCX3 silencing interferes with survival of those cells but does not significantly modify clonogenicity after radiation.

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Disclosure Statement

The authors of this manuscript declare that they have no conflicts of interests.

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