Combined Treatment with Low-Dose Ionizing Radiation and Ketamine Induces Adverse Changes in CA1 Neuronal Structure in Male Murine Hippocampi

Daniela Hladik 1,2, Sonja Buratovic 3, Christine Von Toerne 4, Omid Azimzadeh 1, Prabal Subedi 3, Jos Philipp 1, Stefanie Winkler 1, Annette Feuchtinger 5, Elenore Samson 5, Stefanie M. Hauck 4, Bo Stenerlöw 6, Per Eriksson 3, Michael J. Atkinson 1,2 and Soile Tapio 1,*

1 Institute of Radiation Biology, Helmholtz Zentrum München, German Research Center for Environmental Health GmbH (HMGU), 85764 Neuherberg, Germany; daniela.hladik@helmholtz-muenchen.de (D.H.); omid.azimzadeh@helmholtz-muenchen.de (O.A.); prabal.subedi@helmholtz-muenchen.de (P.S.); jos.philipp@helmholtz-muenchen.de (J.P.); stefanie.winkler@helmholtz-muenchen.de (S.W.); atkinson@helmholtz-muenchen.de (M.J.A.)
2 Chair of Radiation Biology, Technical University Munich (TUM), 80333 Munich, Germany
3 Department of Environmental Toxicology, University Uppsala, 75236 Uppsala, Sweden; sonja.buratovic@ebc.uu.se (S.B.); per.eriksson@ebc.uu.se (P.E.)
4 Research Unit Protein Science, Helmholtz Zentrum München, German Research Center for Environmental Health GmbH (HMGU), 80939 Munich, Germany; vontoerne@helmholtz-muenchen.de (C.v.T.); hauck@helmholtz-muenchen.de (S.M.H.)
5 Research Unit Analytical Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health GmbH (HMGU), 85764 Neuherberg, Germany; annette.feuchtinger@helmholtz-muenchen.de (A.F.); samson@helmholtz-muenchen.de (E.S.)
6 Department of Immunology, Genetics and Pathology, Uppsala University, 75185 Uppsala, Sweden; bo.stenerlow@igp.uu.se
* Correspondence: soile.tapio@helmholtz-muenchen.de; Tel.: +49-89-3187-3445

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Abstract: In children, ketamine sedation is often used during radiological procedures. Combined exposure of ketamine and radiation at doses that alone did not affect learning and memory induced permanent cognitive impairment in mice. The aim of this study was to elucidate the mechanism behind this adverse outcome. Neonatal male NMRI mice were administered ketamine (7.5 mg kg⁻¹) and irradiated (whole-body, 100 mGy or 200 mGy, \(^{137}\)Cs) one hour after ketamine exposure on postnatal day 10. The control mice were injected with saline and sham-irradiated. The hippocampi were analyzed using label-free proteomics, immunoblotting, and Golgi staining of CA1 neurons six months after treatment. Mice co-exposed to ketamine and low-dose radiation showed alterations in hippocampal proteins related to neuronal shaping and synaptic plasticity. The expression of brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein, and postsynaptic density protein 95 were significantly altered only after the combined treatment (100 mGy or 200 mGy combined with ketamine, respectively). Increased numbers of basal dendrites and branching were observed only after the co-exposure, thereby constituting a possible reason for the displayed alterations in behavior. These data suggest that the risk of radiation-induced neurotoxic effects in the pediatric population may be underestimated if based only on the radiation dose.

Keywords: hippocampus; proteomics; BDNF; CA1 neurons; dendrite abnormality; Golgi staining; irradiation
1. Introduction

Ionizing radiation is an integral part of medical treatment and diagnostics. In radiotherapy, healthy tissues outside the target volume are exposed to low-dose radiation. Therefore, the possibility of radiation-associated risks must be considered. This is especially so for children, since exposure to low doses of ionizing radiation is associated with increased risk of malignancies and cognitive impairment later in life [1–3].

In paediatric radiotherapy and imaging, sedation is often applied during imaging to ensure immobilization [4,5]. Ketamine exerts seductive properties via blockage of the N-methyl-D-aspartate (NMDA) receptors in the brain. These ionotropic glutamate receptors are involved in synaptic plasticity, learning, and memory [6–8]. Ketamine exposure during early brain development has been shown to induce neurodegeneration, followed by cognitive impairment [9–11].

The effects of combined exposure to clinically relevant doses of ketamine (7.5 mg kg$^{-1}$ body weight) and whole-body low-dose radiation (100 mGy, 200 mGy) during brain development were studied in mice [12]. The co-exposed mice showed lack of habituation, hyperactivity, and reduced learning and memory capabilities, while mice exposed to single agents showed no significant differences in behavior compared to non-exposed controls. A combination of drug and irradiation consistently impaired cognitive function [12].

The aim of this study was to elucidate molecular mechanisms that could be associated with the previously observed cognitional impairment. Therefore, we used mice that were treated identically to the previous experiment [12]. We found that the combination of low-dose radiation and ketamine consistently changed the hippocampal proteome. The combination treatment altered the structure of CA1 neurons, while individual treatments did not display this effect. Neuronal morphology, such as dendrite complexity and spin density, are strongly correlated with neuronal function. Therefore, these observations provide a plausible mechanistic reasoning for the detrimental interaction of ketamine and radiation in the developing brain of newborn mammals.

2. Results

2.1. Analysis of the Hippocampal Proteome after Single or Combined Treatment

The hippocampal proteomes of all of the mice were analyzed using label-free LC/MS-MS. In total, 2668 proteins were identified, of which 1839 were quantified based on at least two unique peptides (UP) (Tables S1–S5). Volcano plots of all quantified proteins showed the distribution of nonregulated and deregulated proteins (Figure 1B). Using the filtering criteria (identification with two UP, $p \leq 0.05$, fold-change ±1.3) the analysis showed the following numbers of significantly deregulated proteins in comparison to the control group: 103 in the ketamine (Ket) group, 144 in the 100 mGy group, 122 in the 200 mGy group, 164 in the 100 mGy Ket group, and 157 in the 200 mGy Ket group (Figure 1C). Shared deregulated proteins in the different experimental groups are shown in the Venn diagram in Figure 1D. The two co-exposure groups shared 55 deregulated proteins (Figure 1D). These are listed alongside the fold-changes and GO biological functions in Table 1. The majority of these proteins were classified as members of actin cytoskeleton organization or neuronal development.
Figure 1. Changes in the hippocampal proteome after single treatment or co-treatment. (A) Schematic presentation of the experimental groups and the treatment schedule. (B) Volcano plots representing the distribution of all quantified proteins (identification with at least two UP) in hippocampi exposed to single treatment with ketamine (Ket), gamma radiation (100 mGy, 200 mGy), or combined treatment (100 mGy Ket, 200 mGy Ket). Deregulated proteins ($p \leq 0.05$, fold-change $\pm 1.3$) are highlighted in green (downregulated) and red (upregulated). (C) Total numbers of significantly downregulated (green) and upregulated (red) proteins are shown for all treatments ($p \leq 0.05$, fold-change $\pm 1.3$). (D) Venn diagram illustrating the number of shared deregulated proteins between the five experimental groups.
Table 1. Significantly deregulated proteins shared in the combined treatment groups with ketamine and irradiation.

| Symbol | Entrez Gene Name                        | Fold-Change 100 mGy Ket | Fold-Change 200 mGy Ket | Biological Function                                         | GO Number       |
|--------|----------------------------------------|-------------------------|-------------------------|------------------------------------------------------------|-----------------|
| 1      | ABHD10 abhydrolase domain containing 10 | -2.014                  | -1.602                  | glucuronoside catabolic process                             | GO:0019391      |
| 2      | ACAN aggrecan                           | -2.742                  | -3.063                  | negative regulation of cell migration                      | GO:0030336      |
| 3      | ADAM11 ADAM metalloproteinase domain 11 | -1.344                  | -1.622                  | proteolysis                                                | GO:0006508      |
| 4      | ADAM23 ADAM metalloproteinase domain 23 | -1.325                  | -1.409                  | proteolysis                                                | GO:0006508      |
| 5      | ARF6 ADP ribosylation factor 6          | -1.432                  | -1.365                  | regulation of dendritic spine development                  | GO:0069998      |
| 6      | ARMC1 armadillo repeat containing 1     | -2.059                  | -1.752                  | metal ion transport                                        | GO:0030001      |
| 7      | ARPC1A actin related protein 2/3 complex subunit 1A | 1.358                | 1.515                   | regulation of actin filament polymerization                | GO:0030833      |
| 8      | ASPA aspartoacylase                     | 1.406                   | 1.575                   | positive regulation of oligodendrocyte differentiation      | GO:0048714      |
| 9      | BRSK2 BR serine/threonine kinase 2      | -1.680                  | -1.829                  | neuron differentiation                                      | GO:0030182      |
| 10     | CBR3 carbonyl reductase 3               | -1.478                  | -1.577                  | cognition                                                  | GO:0050890      |
| 11     | CDC42 cell division cycle 42            | -1.588                  | -1.474                  | modification of synaptic structure                         | GO:0099563      |
| 12     | CRK CRK proto-oncogene. adaptor protein  | -1.948                  | -2.240                  | dendrite development                                       | GO:0016358      |
| 13     | DNAJC6 DnaJ heat shock protein family (Hsp40) member C6 | -1.381                | -1.484                  | synaptic vesicle uncoating                                 | GO:0016191      |
| 14     | DYNLL2 dynein light chain LC8-type 2    | -1.316                  | -1.329                  | microtubule-based process                                  | GO:0007017      |
| 15     | ELMO2 engulfment and cell motility 2    | -2.612                  | -2.402                  | cytoskeleton organization                                  | GO:0007010      |
| 16     | FBXO2 F-box protein 2                   | -1.503                  | -1.307                  | regulation of protein ubiquitination                       | GO:0031396      |
| 17     | GDPD1 glycerophosphodiester phosphodiesterase domain containing 1 | -1.286                | -1.293                  | N-acylcholinolamine metabolic process                      | GO:0070291      |
| 18     | GGT7 gamma-glutamyltransferase 7        | -2.601                  | -4.805                  | regulation of response to oxidative stress                 | GO:1902883      |
| 19     | GUK1 guanylate kinase 1                 | -1.496                  | -1.412                  | ATP metabolic process                                      | GO:0046034      |
| 20     | HIST1H2BD histone cluster 1 H2B family member d | 1.562                | 1.515                   | protein ubiquitination                                     | GO:0016567      |
| 21     | HNRNPU1 heterogeneous nuclear ribonucleoprotein U like 1 | -3.209                | -1.488                  | RNA processing                                             | GO:0006396      |
| 22     | HTT huntingtin                         | -1.862                  | -1.686                  | learning or memory                                         | GO:0007611      |
| 23     | IPO5 importin 5                        | -1.568                  | -1.478                  | protein import into nucleus                                | GO:0006606      |
| 24     | MICU3 mitochondrial calcium uptake family member 3 | -1.285                | -1.484                  | mitochondrial calcium ion transmembrane transport           | GO:006851       |
| 25     | NACA nascent polypeptide associated complex subunit alpha | -1.297                | -1.290                  | positive regulation of nucleic acid-templated transcription | GO:1903508      |
Table 1. Cont.

| Symbol | Entrez Gene Name | Fold-Change 100 mGy Ket | Fold-Change 200 mGy Ket | Biological Function | GO Number |
|--------|------------------|-------------------------|-------------------------|---------------------|------------|
| 26     | NDRG2            | −1.295                  | −1.326                  | nervous system development | GO:0001818 |
| 27     | NIF3L1           | −1.649                  | −1.671                  | neuron differentiation | GO:0030182 |
| 28     | NRP1             | −1.935                  | −2.487                  | axon guidance        | GO:0007411 |
| 29     | OCIAD1           | 1.529                   | 1.818                   | regulation of stem cell differentiation | GO:2000736 |
| 30     | PAK3             | −2.309                  | −2.063                  | dendritic spine development | GO:0006996 |
| 31     | PCDH1            | −1.874                  | −3.052                  | cell adhesion        | GO:0007155 |
| 32     | PFDN6            | −2.242                  | −1.669                  | protein folding      | GO:0006457 |
| 33     | PIP5K1C          | −1.286                  | −1.306                  | axonogenesis         | GO:0007409 |
| 34     | PRKAR2A          | −1.292                  | −1.351                  | modulation of chemical synaptic transmission | GO:0050804 |
| 35     | PTGES3           | −1.543                  | −1.780                  | prostaglandin biosynthetic process | GO:0001516 |
| 36     | PTPRS            | −1.545                  | −1.301                  | hippocampus development | GO:0021766 |
| 37     | RAB1A            | −1.302                  | −1.374                  | intracellular protein transport | GO:0006886 |
| 38     | RAB5C            | −1.323                  | −1.423                  | intracellular protein transport | GO:0006886 |
| 39     | RABL6            | 1.737                   | 1.548                   | intracellular protein transport | GO:0006886 |
| 40     | RIMBP2           | −1.385                  | −1.856                  | neuromuscular synaptic transmission | GO:0007274 |
| 41     | RPLP2            | −1.543                  | −1.508                  | translational elongation | GO:0006414 |
| 42     | SEC24C           | −1.342                  | −1.390                  | vesicle-mediated transport | GO:0016192 |
| 43     | SLC1A4           | −1.414                  | −1.344                  | cognition             | GO:0050890 |
| 44     | SNCA             | −1.432                  | −1.421                  | synaptic transmission | GO:0001963 |
| 45     | STX7             | −1.324                  | −1.298                  | vesicle-mediated transport | GO:0016192 |
| 46     | SUCLG1           | 1.342                   | 1.356                   | succinyl-CoA metabolic process | GO:0006104 |
| 47     | TIMM13           | −1.845                  | −1.487                  | protein insertion into mitochondrial inner membrane | GO:0045039 |
| 48     | TPDS2            | −1.468                  | −1.384                  | positive regulation of cell population proliferation | GO:0008284 |
| 49     | TRAPPC10         | −1.392                  | −1.664                  | vesicle-mediated transport | GO:0016192 |
| 50     | TRIO             | −1.562                  | −1.369                  | G-protein-coupled receptor signaling pathway | GO:0007186 |
Table 1. Cont.

| Symbol | Entrez Gene Name | Fold-Change 100 mGy Ket | Fold-Change 200 mGy Ket | Biological Function | GO Number |
|--------|------------------|--------------------------|------------------------|---------------------|-----------|
| 51     | TUBA8            | −1.472                   | −1.343                 | microtubule cytoskeleton organization | GO:0000226 |
| 52     | UBXN6            | −1.360                   | −1.419                 | macroautophagy       | GO:0016236 |
| 53     | UCHL3            | −1.413                   | −1.427                 | adult walking behavior | GO:0007628 |
| 54     | VBP1             | −1.747                   | −1.809                 | protein folding      | GO:0006457 |
| 55     | WASF3            | −1.904                   | −1.429                 | actin cytoskeleton organization | GO:00300036 |

2.2. Effects on Neuronal Cytoskeleton and Synaptic Plasticity Following Combined Exposure to Ketamine and Irradiation

To better understand the involvement of biological processes following the combined treatment with ketamine and radiation, the 55 common significantly deregulated proteins from the co-exposure groups were subjected to Ingenuity Pathway Analysis (IPA). In particular, the categories “canonical pathways” and “diseases and biofunctions” were analyzed (Figure 2A). The most enriched canonical pathways were involved either in the organization of the cytoskeleton (signaling by Rho GDI family GTPases, RHOGDI signaling, actin cytoskeleton signaling, Rac signaling, RhoA signaling, regulation of actin-based motility by Rho) or played a role in neuronal transmission (ephrin receptor signaling, cAMP-mediated signaling, integrin signaling). Similarly, the most affected biofunctions were related to reorganization of the neuronal structure (shape change of axons, axonogenesis, growth of neurites, branching of axons) and synaptic transmission (transport of synaptic vesicles, neurotransmission, synaptic depression, long-term potentiation) (Figure 2A).

Activation of the brain-derived neurotrophic factor (BDNF) was predicted based on the deregulation profiles of the co-exposed groups by IPA (Figure 2B). BDNF is one of the key regulators of neuronal morphology and stimulates the growth and differentiation of new neurons and synapses [13]. In good agreement with this, the level of BDNF investigated by immunoblotting showed a significant increase in its expression in the co-exposed groups (upregulation by mean fold-changes of 4.2 (p < 0.001) and 3.6 (p < 0.01) in the groups “100 mGy Ket” and “200 mGy Ket”, respectively) (Figure 2C).
2.3. Morphological Abnormalities of Hippocampal CA1 Neurons only after Combined Treatment with Ketamine and Irradiation

Golgi-Cox staining followed by dendritic reconstruction was performed on tissue sections of the Cornu Ammonis (CA1). Raw images are presented exemplarily for every experimental condition in Figure S2. Representative images of reconstructed neurons are shown in Figure 3A. Apical and basal dendrites were analyzed separately in all experimental groups (Figure 3B). No effect was found on the...
structure and number of apical parts of the CA1 neurons (Figure S3A–C). A significant increase ($p < 0.001$) in the total number of basal dendrites was present after the combined treatment with ketamine and radiation (Figure 3B). In the co-treated groups, the total number of nodes was significantly increased (100 mGy Ket: $p < 0.05$, 200 mGy Ket: $p < 0.001$) while there was no difference in the single treatment groups compared to the control (Figure 3C). The number of spines divided by the dendrite length was significantly reduced in the group co-exposed to 200 mGy Ket ($p < 0.05$) (Figure 3D). However, the reduction of spines was not significant in the 100 mGy Ket group and therefore could not explain the observed cognitional impairment. No effect in spine number was detected in the apical dendrites (Figure S3C). This indicated an increase in the complexity of the basal dendrites after co-exposure.

**Figure 3.** Co-treatment with ketamine and irradiation led to structural changes in hippocampal CA1 neurons. (A) Reconstructed hippocampal CA1 neurons representative for each experimental treatment group are shown. Each individual dendrite is presented in a different color. (B) The number of basal dendrites is shown in all experimental groups. Five neurons per animal with 5 biological replicates were analyzed; ***$p < 0.001$. (C) The number of nodes representing the branching points in basal dendrites is shown. Five neurons per animal with 5 biological replicates were analyzed; *$p < 0.05$, ***$p < 0.001$. (D) The spine densities of the basal dendrites are shown. Five neurons per animal with
5 biological replicates were analyzed; * p < 0.05, *** p < 0.001. (D) The spine densities of the basal dendrites are shown. Five neurons per animal with 5 biological replicates were analyzed; * p < 0.05. (E) A comparison between the total number of dendritic intersections for each circle between the controls (black) and the co-treated neurons (100 mGy Ket: blue; 200 mGy Ket: purple) was performed. The co-treated animals showed significant increase in the number of intersections between 40 and 80 µm (100 mGy ket; + p < 0.05, ++ p < 0.01, +++ p < 0.01) and between 20 and 70 µm (200 mGy Ket; * p < 0.05, ** p < 0.01, *** p < 0.001). At least 5 neurons per animal with 5 biological replicates were analyzed. The first values (2 µm circle) represent the total number of basal dendrites, as shown in (B). (F) Representative CA1 neurons with concentric circles used for the Sholl analysis are shown. The radius interval between the circles was set to 10 µm per step, ranging from 10 to 200 µm from the center of the neuronal soma to the end of the dendrites. The numbers of dendritic intersections per circle were quantified. At least 5 neurons were analyzed per animal. The p values were calculated using a two-way ANOVA with Bonferroni multiple testing.

To investigate this in more detail, Sholl analysis, representing the distribution of dendritic intersections with increasing distance from the cell soma, was performed. A significant shift in the number of intersections in the circle diameter of 20–80 µm was observed in the co-exposed groups compared to the sham-treated controls, thereby confirming a significant increase in the number of basal dendrites and their branching points (Figure 3E,F).

3. Discussion

Pediatric radiotherapy and treatment frequently require ketamine sedation prior to irradiation [15]. Concerns about the long-term safety of this combination have been raised following the report of cognitive impairment in mice co-exposed to clinically relevant doses of ketamine and irradiation [12]. We show here that co-exposure during early brain development results in persistent alterations to both the proteome and structure of hippocampal CA1 neurons. Significant increases in dendrite number and branching were observed when ketamine was given immediately prior to irradiation at 100 or 200 mGy. Neither ketamine nor radiation treatment alone induced the reorganization of dendritic structures.

The structure of dendrites has a profound impact on the processing of neuronal information, including learning and memory. The formation of the dendritic arbour, the sides of synaptic connections from input neurons, is usually completed by adulthood. Aberrations and remodeling of dendritic structures are observed only under pathological conditions [16–18]. Extension of the dendrite length beyond the normal level is related to mental retardation [19,20]. Neuropathic pain linked to depression and cognitive decline is known to be associated with an increase in dendritic length and branching [21].

Interestingly, ketamine (10 µM) was previously shown to promote both the number of dendritic branches and the total length of the arbours in embryonic rat cortical neurons in vitro [22] due to ketamine-induced rapid increase in BDNF secretion [23,24]. Cranial irradiation of adult mice using radiation doses higher than in this study (1 or 10 Gy) resulted in significant reductions in dendritic branching and total length in the hippocampus [25]. Our data showed increased basal branching of CA1 neurons with the combined exposure, therefore suggesting a radiation-enhanced ketamine-like response in the neuronal structure and demonstrating the strong impact of ketamine and irradiation when applied together.

In contrast, the regulation of neuronal structures is dependent upon several factors. Small GTP binding proteins, like Ras-related C3 botulinum toxin substrate 1 (RAC1), Ras homolog gene family member A (RHOA), and cell division control protein 42 homolog (CDC42), are crucial for reorganization of the dendrites and their branching [26–29]. In accordance with this, our proteome analysis showed that most of the significantly deregulated proteins in the co-exposed groups belonged to pathways dependent on the Rho family GTPases. Thus, changes in the neuronal cytoskeleton and associated pathways (shape change of axons, axonogenesis, growth of neurites, and branching of axons) were all predicted from the proteome data and were consistent with the changes in CA1
neuronal morphology. Secreted factors such as neurotrophins are known to play key roles in regulating dendrite outgrowth and branching [30]. BDNF is a well-studied mediator of synaptic plasticity and memory formation [31,32]. Overexpression of BDNF in CA1 neurons was shown to improve fear and object-location memory in mice [33], but was associated with cognitive impairment in MECP2-duplication syndrome [34]. Application of exogenous BDNF was shown to increase the number of dendrites in pyramidal neurons [35]. Ketamine given at doses of 10 or 15 mg kg\(^{-1}\) (but not at 5 mg kg\(^{-1}\)) resulted in a rapid and significant increase in the expression of hippocampal BDNF in adult male Wistar rats [36]. In contrast, high-dose cranial irradiation (10 Gy) of adult C57BL/6 mice resulted in a significant decrease in BDNF expression in the hippocampus one month after exposure [37]. In our study, only the co-treatment with ketamine (7.5 mg kg\(^{-1}\)) and low-dose radiation (100 mGy or 200 mGy) led to an increase in the total amount of BDNF in the hippocampus. This increase was sustained long after exposure in neonatal mice.

Similar to BDNF, PSD-95 is involved in synaptic functions, especially with regard to NMDA receptors [38,39]. We previously showed that whole-body irradiation of neonatal NMRI or C57BL/6J mice causes increased expression of PSD-95 in the hippocampus six months after exposure [40,41]. This was seen at whole-body doses equal to or higher than 0.5 Gy, but not at lower doses. Similarly, it was shown that high-dose (1 Gy, 10 Gy) cranial gamma-radiation caused increased expression of PSD-95 in the hippocampus of adult mice 30 days after irradiation [25]. Administration of high-dose ketamine (30 mg kg\(^{-1}\) over five consecutive days) was shown to immediately increase the level of PSD-95 in the synaptosomes of adult male Wistar rats [42]. Lower doses of ketamine (10 mg kg\(^{-1}\)) did not produce effects on the total level of PSD-95 in the hippocampal membranes of mice immediately (30 min) after administration [43]. In addition to synaptic functions, both BDNF and PSD-95 play a role in regulating dendrite outgrowth and branching [14,35]. In contrast to BDNF, PSD-95 inhibits the branching of dendrites [14]. Contrary to our previous results obtained using a less-sensitive slot blotting method [12], the immunoblotting used here showed reduced expression of PSD-95 after the combined treatment with ketamine and low-dose radiation. No change in the PSD-95 level was detected using single exposure treatments. The persistent upregulation of BDNF and downregulation of PSD-95 after the co-exposure, in addition to the changes in the expression levels of several other proteins responsible for neuronal growth and branching (CDC42, PAK3, ARF6, and others displayed in Table 1), could be the molecular explanation for the observed increase in the number and branching of basal dendrites in the CA1 neurons.

4. Materials and Methods

4.1. Animals

All procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC; approval date: 26 April 2013) after permission by local ethical committees (Uppsala University) and the Swedish Committee for Ethical Experiments on Laboratory Animals. The results were reported in line with relevant aspects of the ARRIVE guidelines [44].

Pregnant Naval Medical Research Institute (NMRI) mice were purchased from Scanbur (Sollentuna, Sweden) and housed in Makrolon® III cages. Only the male offspring were used in the experiments to mimic the conditions used for cognitional testing [12].

4.2. Exposure

Neonatal (postnatal day 10) mice were exposed to a single subcutaneous injection of ketamine (7.5 mg kg\(^{-1}\) body weight) or to a low dose of whole-body gamma radiation (\(^{137}\)Cs; 100 mGy, 200 mGy), or co-exposed. In the co-exposure group, ketamine was administered one hour before irradiation. Control mice were injected with 10 mL kg\(^{-1}\) body weight saline (0.9%) and sham-irradiated. Control mice and mice irradiated only were not given sedatives prior to exposure. The dosages of ketamine and irradiation were determined based on the previous experiments showing no effect of single exposures
on spontaneous behavior, learning and memory, or protein levels [12,45–47]. A schematic presentation of the experimental design is shown in Figure 1A.

4.3. Tissue Collection

The mice were sacrificed with CO2 6 months post-treatment. Brains were excised, dissected, and rinsed in cold PBS. The right hemisphere was used for proteome analysis. The hippocampi were microdissected, snap frozen in liquid nitrogen, and stored at −80 °C. The left hemisphere was used for the morphology study.

4.4. Protein Lysis and Determination of Protein Concentration

Frozen hippocampi were pulverized and suspended in RIPA buffer (Thermo Fischer, Darmstadt, Germany) enriched with phosphatase and protease inhibitors (Sigma-Aldrich, Taufkirchen, Germany).

After sonication, lysis, and centrifugation, protein concentrations were measured using BCA Protein Assay Kit (Thermo Fischer) according to the manufacturer’s instructions.

4.5. Mass Spectrometry (MS)

Label-free measurements were performed on a QExactive high field mass spectrometer (Thermo Fisher) in data-dependent acquisition mode, as described previously [48,49].

4.6. Protein Identification and Quantification

Spectra were analyzed using Progenesis QI software (Version 3.0, Nonlinear Dynamics) for label-free quantification, as described before [48]. The filtering criteria were as follows: Proteins identified and quantified with two UP and fold-changes of ≤0.77 or ≥1.3 (t-test; p ≤ 0.05) were considered to be significantly differentially expressed.

4.7. Pathway Analysis

The list of significantly deregulated proteins with their accession numbers, fold-changes and p-values were imported into Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity).

4.8. Western Blot Analysis

Western blots were performed according to the protocol described previously [48]. The following antibodies were used: BDNF (abcam, ab203573, Cambridge, UK), ARC (abcam, ab118929, Cambridge, UK) and PSD-95 (abcam, ab18258, Cambridge, UK). Ponceau (Sigma-Aldrich, St. Louis, MO, USA) staining served as an internal loading control (Figure S1).

4.9. Golgi Staining

Staining was performed using the FD Rapid GolgiStain Kit (NeuroTechnologies, Columbia, SC, USA) according to the user manual. Directly after dissection, the hemispheres were put into 5 mL Golgi-Cox solution for fixation and impregnation for one week and then frozen at −20 °C. For imaging, the frozen brain was cut with a cryostat at −20 °C (100 µm coronal sections), and the sections were mounted on gelatin-coated microscope slides.

4.10. Imaging and Analysis of Dendrites and Spines

CA1 neurons were reconstructed using Neurolucida software (MBF Bioscience, Williston, ND, USA) according to the user manual. Directly after dissection, the hemispheres were put into 5 mL Golgi-Cox solution for fixation and impregnation for one week and then frozen at −20 °C. For imaging, the frozen brain was cut with a cryostat at −20 °C (100 µm coronal sections), and the sections were mounted on gelatin-coated microscope slides. In the Sholl analysis, the radius interval of each section was set to 10 µm, starting from 10 µm and ending at a 200 µm distance from the soma.
4.11. Statistical Analysis

Statistical analysis of the LC-MS/MS data was performed with Excel using a two-sided Student’s t-test. The Western blotting and the Golgi-Cox assay were analyzed using GraphPad prism software (GraphPad Software, San Diego, CA, USA) and a 2-way ANOVA with Bonferroni multiple testing. The error bars were calculated as standard error of the mean (SEM); \( p \)-values \( \leq 0.05 \) were defined as significant.

4.12. Data Availability

The raw MS-data are available at http://dx.doi.org/doi:10.20348/STOREDB/1132/1198.

5. Conclusions

In conclusion, the data from this study corroborated the results from the previous study regarding behavioral effects [12]. Both studies strongly suggested that a scenario of early postnatal exposure to a combination of ketamine and low-dose radiation, comparable to that found in clinical situations, was able to persistently induce cognitive impairment and changes in the neuronal structure. The neonatal window used in this study corresponded to the human brain developmental period that starts around the third trimester of pregnancy and expands over the first two years of life. Considering that ketamine is one of the most commonly used sedative agents in pediatric emergency departments [50], these results raise concern over the detrimental long-term effects on cognitive function. Whether the combination of ketamine and low-dose radiation is able to induce and exacerbate developmental neurobehavioral and cognitive defects in children should be investigated further, as this may be highly relevant for daily clinical practice.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/23/6103/s1. Table S1. All quantified proteins with an identification based on at least two UP for the ketamine group. Table S2. All quantified proteins with an identification based on at least two UP for the 100 mGy group. Table S3. All quantified proteins with an identification based on at least two UP for the 200 mGy group. Table S4. All quantified proteins with an identification based on at least two UP for the 100 mGy Ket group. Table S5. All quantified proteins with an identification based on at least two UP for the 200 mGy Ket group. Figure S1. Ponceau staining and western blot bands. Figure S2. Representative hippocampal CA1 neurons for all experimental groups. Figure S3. Co-treatment with ketamine and irradiation does not affect the number of the apical dendrites, their branching or the spine density.

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