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ORIGINAL PAPER

Risperidone and haloperidol promote survival of stem cells in the rat hippocampus

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Abstract Altered neuroplasticity contributes to the pathophysiology of schizophrenia. However, the idea that antipsychotics may act, at least in part, by normalizing neurogenesis has not been consistently supported. Our study seeks to determine whether hippocampal cell proliferation is altered in adult rats pretreated with ketamine, a validated model of schizophrenia, and whether chronic administration with neuroleptic drugs (haloperidol and risperidone) affect changes of cell genesis/survival. Ketamine per se has no effect on cell proliferation. Its withdrawal, however, significantly induced cell proliferation/survival in the hippocampus. Risperidone and haloperidol supported cell genesis/survival as well. During ketamine withdrawal, however, their application did not affect cell proliferation/survival additionally. TUNEL staining indicated a cell-protective potency of both neuroleptics with respect to a ketamine-induced cell death. As RT-PCR and Western blot revealed that the treatment effects of risperidone and haloperidol seemed to be mediated through activation of VEGF and MMP2. The mRNA expression of NGF, BDNF, and NT3 was unaffected. From the respective receptors, only TrkA was enhanced when ketamine withdrawal was combined with risperidone or haloperidol. Risperidone also induced BCL-2. Ketamine withdrawal has no effect on the expression of VEGF, MMP2, or BCL-2. It activated the expression of BDNF. This effect was normalized by risperidone or haloperidol. The findings indicate a promoting effect of risperidone and haloperidol on survival of young neurons in the hippocampus by enhancing the expression of the anti-apoptotic protein BCL-2 and by activation of VEGF/MMP2, whereby an interference with ketamine and thus a priority role of the NMDA system was not evident.

Keywords Animal model · BrdU · Ketamine · Schizophrenia

Abbreviations

5-HT2A	5-Hydroxytryptamine (serotonin) receptor 2A
BCL-2	B cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BrdU	5-Bromo-2′-deoxy-uridine
cAMP	Cyclic adenosine-3′,5′-monophosphate
cDNA	Complementary DNA
CREB	cAMP response element binding protein
CTGF	Connective tissue growth factor
DCX	Doublecortin
dUTP	2′-Deoxyuridine 5′-triphosphate
ERK	Extracellular signal-regulated kinase
Galac	Galactocerebroside
GAPDH	Glycerinaldehyde-3-phosphate-dehydrogenase
GFAP	Glial fibrillary acidic protein
HARP	Heparin affin regulatory peptide

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i.p.	Intraperitoneally
JNK	c Jun-N-terminal kinase
K/H	Ketamine/haloperidol
K/R	Ketamine/risperidone
K/S	Ketamine/saline
LSAB	Labeled streptavidin biotin horseradish peroxidase
MEK	Mitogen-activated protein (MAP) kinase
MMP2	Matrix metallopeptidase 2 (gelatinase A)
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NT3	Neurotrophin
OX42	Integrin alpha-M
PBS	Phosphate-buffered saline
PCP	Phencyclidine
PI3K	Phosphatidylinositol-3-kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
S/H	Saline/haloperidol
S/R	Saline/risperidone
S/S	Saline/saline
TBS	Tris-buffered saline
TdT	Terminal deoxynucleotidyl transferase
Trk	Tyrosine kinase receptor
TUNEL	Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling
VEGF	Vascular endothelial growth factor

Introduction

Developmental dysfunction of the hippocampus is thought to play a major role in the pathogenesis of schizophrenia [20, 46]. Defects such as reduction of hippocampal volume [14, 22], hippocampal shape deformation, or abnormalities in the hippocampal cell density [21] have been reported. This and the characteristic age at onset of schizophrenia, late adolescence to young adulthood, led to the idea that adult neurogenesis may be disturbed in schizophrenia [43]. Neurogenesis-relevant research on postmortem human tissue has just begun. Reif and co-workers [42] were able to demonstrate that the first step of adult neurogenesis, cell proliferation, is diminished in the dentate gyrus of patients suffering from schizophrenia. Moreover, the effects of typical and atypical antipsychotics on proliferation and neurogenesis have been investigated repeatedly (reviewed by [50]). However, the idea that antipsychotics may act, at least in part, by increasing neurogenesis has not been consistently supported. Six studies (reviewed in [38]) with acute and chronic treatments of haloperidol showed unaltered neurogenesis and cell proliferation in the dentate gyrus, whereas only in the study of Dawirs et al. [11] a positive effect on granule cell proliferation in the gerbil hippocampus after application of an exorbitant high dose of four times 5 mg/kg haloperidol has been demonstrated. Olanzapine increased cell proliferation in the subventricular zone but not in the dentate gyrus [19], whereas results obtained after risperidone treatment are not uniform: in the hands of Green an co-workers [19], it was ineffective in the dentate gyrus and in the subventricular zone as well; by others, however, it increased the number of newly divided cells in the subventricular zone [51]. Moreover, olanzapine but not risperidone increased the numbers of dividing cells within the prefrontal cortex [19].

However, overall findings with neuroleptics were not as robust as those for antidepressant drugs [15, 38]. Differences may be due to diversity in the type of neuroleptics used, dosage and application regime, or animal models. Thus, we have found that subchronic administration of subanesthetic doses of the non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine increased cell proliferation/survival in the dentate gyrus of rats [31]. This treatment has been reported to produce positive and negative symptoms and cognitive impairments consistent with those seen in schizophrenia, hence representing a valid model in experimental schizophrenia research [3, 4, 28, 34]. Liu and co-workers [35] have demonstrated that phencyclidine, another non-competitive NMDA receptor antagonist that triggered schizophrenia-like symptoms similar to ketamine, decreased cell proliferation in the dentate gyrus, but only transiently.

Thus, further work is required to sort out these discrepancies. This prompted us to ask whether chronic administration of neuroleptics affect induced neurogenesis/cell survival in the ketamine model of schizophrenia. The traditional drug haloperidol and the atypical neuroleptic risperidone have been selected for these experiments. From previous studies, it is known that risperidone effectively counteracts ketamine-induced alterations in social behavior, whereas haloperidol seems to be unable to normalize the behavioral changes in this animal model [3]. This may be due to different modes of action. Both neuroleptics act on multiple neurotransmitter receptors, although each drug can be characterized by its specific receptor-binding profile. Haloperidol acts primarily on dopamine D2 receptors with lower activity at D1, D3, D4, 5-HT2A, and α1 adrenergic receptors. Risperidone possesses high serotonin (5-HT2A) receptor antagonism combined with relatively weak D2 receptor antagonism [6, 10, 47, 48], seeming to be relevant for improving neurocognitive dysfunction of risperidone-treated patients [44].

Two major components of neurogenesis are generally measured: the number of newly proliferated cells produced and the number of cells surviving to later time points. In

the following experiments, we examined the effect of haloperidol and risperidone alone and in combination with ketamine pretreatment on cell survival in the subgranular cell layer of the dentate gyrus of the adult rat hippocampus.

Experimental procedures

All experiments were performed in strict accordance with principles of laboratory animal care and the German law of the Protection of Animals and EC guidelines. The present investigations were carried out using male Sprague-Dawley rats (Shoe:Wist/Shoe, DIMED Schönwalde GmbH) aged 8 weeks. The animals were housed in groups of five rats per cage under controlled laboratory conditions (temperature 20 ± 2°C, relative air humidity 55–60%, LD 12:12, lights on at 06:00 a. m.), with free access to standard diet (Altromin 1326) and tap water. Each effort was made to minimize the amount of suffering and the number of animals used in the experiments.

Animal model

As reported previously [3], male Sprague-Dawley rats were pretreated with 30 mg/kg ketamine (CU Chemie Uetikon GmbH, Lahr, Germany) intraperitoneally (i.p.) at a volume of 1 ml/100 g body weight daily for five consecutive days (K/S, K/H, K/R; $n = 9$ per group for decapitation point A, and $n = 15$ per group for decapitation point B; Fig. 1). The substance was freshly prepared and used. Control animals received isotonic saline at corresponding times (S/S, S/H, S/R; $n = 9$ per group for decapitation point A, and $n = 15$ per group for decapitation point B). Following the final injection, the rats were housed singly (Makrolon type III cages with food and water ad libitum). The cages were located together in racks so that auditory and olfactory contact was maintained. During the last 10 days of single housing, the animals received subchronically either haloperidol (S/H, K/H; 0.075 mg/kg, Haldol®, Janssen-Cilag, Neuss, Germany) or risperidone (S/R, K/R; 0.2 mg/kg, Janssen Biotech, Neuss, Germany). Haloperidol was dissolved in isotonic saline. To dissolve risperidone, three drops of Tween 80 were added. Control animals received the solvent in an identical manner. Injections were given i.p. in a volume of 1 ml/100 g body weight. All solutions were freshly prepared before injection. Doses of the neuroleptics are effective to normalize social behavior in ketamine-pretreated rats [3]. The treatment schedule is given in Fig. 1.

Assessment of cell proliferation and identification

To assess cell proliferation, nine rats per group were examined. An immunofluorescence assay for detection of 5-bromo-2′-deoxy-uridine (BrdU, Boehringer Mannheim, Germany) incorporated into cellular DNA in combination with immunofluorescence staining for cell identification was performed as described previously [31]. In brief, rats received one daily i.p. injection of 50 mg/kg BrdU dissolved in physiological saline at days 3–5 of the ketamine-/saline pretreatment. 50 mg/kg (body weight, i.p.) is a common dose used in most studies on adult neurogenesis in rodents (for review see [49]), offering the comparability with our previous and other published results. In this period, volumes of the ketamine and BrdU injections were reduced in such a way that the total volume was 1 ml/100 g body weight.

Twenty-four hours (Fig. 1 decapitation point A, without haloperidol/risperidone treatment) or 3 weeks after the cessation of BrdU treatment (Fig. 1 decapitation point B, 24 h after the last haloperidol/risperidone treatment), animals were anesthetized with an overdose of chloral hydrate and killed by transcardial perfusion (4% 0.1 M phosphate-buffered paraformaldehyde, Merck, Darmstadt, Germany, pH 7.4). Free-floating serial sagittal sections (20 mm thick) were cut on a cryostat (Jung Frigocut 2800 E, Leica, Bensheim, Germany), and selected as shown in Fig. 2. The cryo-sections were incubated in 2 M HCl for DNA denaturation, neutralized with 0.1 M borate buffer, and incubated with a rat monoclonal antibody to BrdU (Oxford Biotechnology Ltd., Oxford, UK, 1:100, in phosphate-buffered saline (PBS) containing 0.3% Triton X-100, Merck, Darmstadt, Germany) for 1 h at 37°C. After several rinses in PBS, slices were incubated with the corresponding antibodies for cell identification: polyclonal rabbit anti-GFAP (Progen, Heidelberg, Germany; 1:50), monoclonal mouse anti-NeuN (Chemicon, Temecula, USA, 1:100), polyclonal goat anti-doublecortin (DCX, Santa Cruz Biotechnology, Santa Cruz, USA; 1:300), monoclonal mouse anti-nestin (Chemicon; 1:100), monoclonal mouse anti-galactocerebroside (Galac, Chemicon; 1:500), or a monoclonal mouse anti-CD 11b/c (Ox42, Pharmingen, Hamburg, Germany; 1:800) in PBS with 0.3% Triton X-100 and

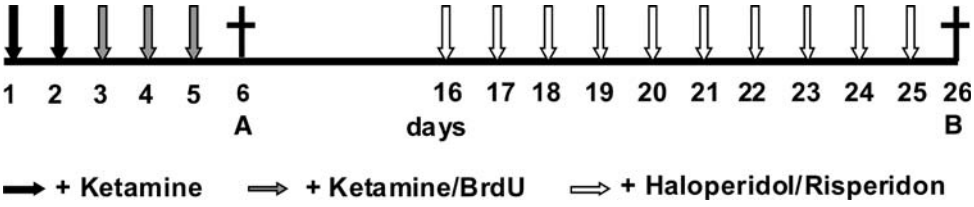

Fig. 1 Treatment schedule

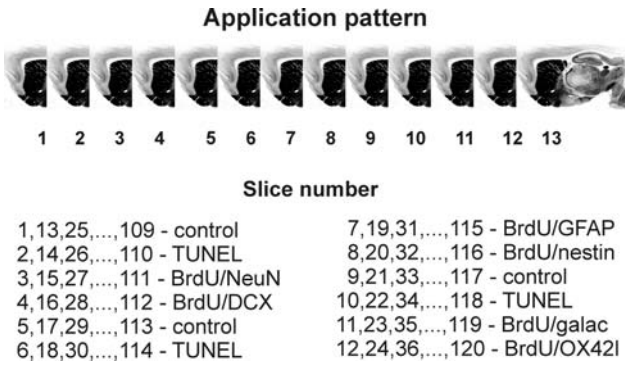

Fig. 2 Application pattern

1% normal goat serum overnight at 4°C. Following this, slices were washed in PBS and incubated overnight with a combination of secondary antibodies (Molecular Probes, Göttingen, Germany; 1:500): goat anti-rat-IgG Alexa Fluor 488/goat anti-rabbit-IgG Alexa Fluor 546 or goat anti-rat-IgG Alexa Fluor 546/goat anti-mouse-IgG Alexa Fluor 488. Some sections were further triple-labeled with DAPI (Sigma, Deisenhofen, Germany; 1 μg/ml) for 10 min to visualize nuclei, then mounted and examined using a fluorescence microscope (Axio Imager.M1, Zeiss, Jena, Germany) equipped with fluorescein, rhodamine, and DAPI optics. Control reactions (substitution of the primary antisera with PBS) yielded negative results (i.e., no specific immunostaining was seen in these sections).

Assessment of cell death

The technique of terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) was used to detect apoptotic DNA fragmentation. Free-floating cryo-sections were washed, permeabilized with methanol:chloroform:acetic acid (66:33:1, 10 min) and incubated in a humidified box (37°C, 90 min) using 50 μl reaction solution: 0.4 μl terminal deoxynucleotidyl transferase (TdT, 0.5 U/μl), 2 μl cobalt chloride (2.5 mM), 4 μl TdT reaction buffer, 0.8 μl biotin-dUTP (2 nM), 12.8 μl MilliQ (Roche Diagnostics, Mannheim, Germany). The reaction was stopped by washing slices in saline sodium citrate (3 × 5 min) and TBS (2 × 5 min). The incorporated dig-dUTP was detected using digoxigenin antibodies with the peroxidase–antiperoxidase technique, and visualized with 3,3′-diaminobenzidine using the DAKO-LSAB-kit (Dako, Hamburg, Germany).

RT-PCR analysis

For RT-PCR analysis, total RNA was isolated from the hippocampus of six animals per group at decapitation point B using guanidinium isothiocyanate/phenol/chloroform (peqGOLD TriFast, peqlab, Erlangen, Germany). To remove contaminating DNA, 5 μg of total cell RNA was treated with Turbo DNA-free (Ambion, Austin, USA) according to the manufacturer's instructions. RNA (4.5 μl; 2.25 μg input RNA) was reverse transcribed using the RevertAid^{TM} H Minus First strand cDNA Synthesis Kit primed with Oligo$(dT)_{18}$ primers (Fermentas, St. Leon-Rot, Germany; primers listed in Table 1). cDNA (2 μl) was then PCR amplified with *Taq*-DNA-polymerase (peqlab). One-tenth of each reaction product was electrophoresed on a 1% agarose gel. The PCR product bands were quantified by densitometric analysis using a Biometra BioDocAnalyzer and the ratio of their expression to expression of the housekeeping gene (GAPDH) was calculated. Before analysis, the quality of RNA has been checked by measuring the UV absorption at 260 and 280 nm. The reliability of GAPDH as reference gene was selected in a comparative pilot study with beta-actin. To ensure that quantification was performed in the exponential phase of the PCR reaction cycle kinetics were performed prior to the analyses. An example of gel image is shown in Fig. 3 (for more detail see [45]).

Western blot analysis

Homogenates were prepared from the same material as mentioned under RT-PCR analysis. Material was mixed with a protein-loading buffer (roti-Load 1, Roth GmbH, Karlsruhe, Germany) according to the manufacturer's procedure and placed in a hot-water bath (95°C) for 5 min. Proteins were separated using SDS-PAGE (gradient gels from 5 to 25%). The amount of protein loaded per lane was 20 μg. After separation, the proteins were transferred to nitrocellulose paper and non-specific protein binding sites were blocked with blocking buffer (Chemicon). The blots were incubated overnight with antibodies to MMP2 (matrix metalloproteinase 2, Chemicon) 1:100, VEGF (vascular endothelial growth factor, recognizing 34–54 kDa isoforms, Chemicon) 1:500, CREB (cAMP response element binding protein, Chemicon) 1:1,000, or phospho-p38 MAPK (R&D Systems, Minneapolis, USA), 1:2,000, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit and anti-mouse IgG^{+} peroxidase, Boehringer GmbH, Mannheim, Germany; 1:10,000). Immunoreactivity was visualized using the ECL detection system (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK) and quantified by densitometric analysis using a Biometra BioDocAnalyzer. In addition, detection of beta-actin was used to show equal sample loading.

Statistical analysis

Unbiased quantification of BrdU- and TUNEL-positive cells was done according to a standard protocol [31]. Two

Table 1 Sequences of primers used for RT-PCR

Gene	Sequence	Location of primers	Product size (bp, base pair)	Cycle no.	Reference (Gene bank Acc. no.)
MMP2	5′-CCCCTATCTACACCTACACCAAGAAC-3′	1,741–1,763	576	30	NM_031054
	5′-CATTCCAGGAGTCTGCGATGAGC-3′	2,291–2,313			
VEGF-A	5′-GAAGTTCATGGACGTCTACC-3′	121–139	237	40	NM_031836
	5′-CATCTCTCCTATGTGCTGGC-3′	357–338			
BCL-2	5′-CGACTTTGCAGAGATGTCCAG-3′	555–575	392	40	NM_016993
	5′-CTCACTTGTGGCCCAGGTATG-3′	946–926			
NGF	5′-AAGGACGCAGCTTTCTATCCTG-3′	145–166	370	40	XM_227525
	5′-CATGGGCCTGGAAGTCTAAATC-3′	514–493			
BDNF	5′-CAGCAGTCAAGTGCCTTTGG-3′	954–973	302	38	NM_012513
	5′-TGCAGCCTTCCTTCGTGTAA-3′	1,255–1,236			
NT-3	5′-GTCGACGTCCCTGGAAATAGTC-3′	250–271	286	40	NM_031073
	5′-TTCTCTGGGTGCCTCTGCTT-3′	534–515			
TrkA	5′-AATGCTCGTCAGGACTTCCATC-3′	1,724–1,745	343	40	NM_021589
	5′-TCTTGACCACTAGTCCCTGACC-3′	2,066–2,045			
TrkB	5′-CGTCACCAATCACACGGAGT– 3′	1,669–1,688	351	35	NM_012731
	5′-AGAAGCAGCATCACCAGCAG-3′	2,019–2,000			
TrkC	5′-TGAGTCTGATGCGAGCCCTA-3′	1,278–1,297	294	40	NM_019248
	5′-CGAGTCATGCCAATGACCAC-3′	1,571–1,552			
GAPDH	5′-TTAGCACCCCTGGCCAAGG-3′	802–820	531	24	XM_228411
	5′-CTTACTCCTTGGAGGCCATG-3′	1,332–1,314			

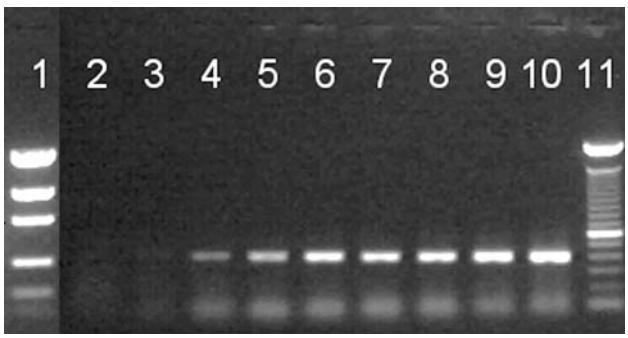

Fig. 3 RT-PCR cycle kinetics of trkA (example gel). Mass ladder (200, 120, 80, 40, 20, and 10 ng cDNA; *lane 1*); RT-PCR with 24, 26, 28, 30, 32, 34, 36, 38 and 40 cycles and annealing temperature 49°C (*lanes 2–10*); 100 bp DNA ladder (*lane 11*)

independent investigators, blinded to the experimental protocol, counted all cell profiles with a clear respective staining using a modified optical dissector method [5]. The complete structure of the dentate gyrus was examined. Therefore, for each animal and each labeling experiment, ten sections, selected as shown in Fig. 2, were scanned in image field by image field using the Axio Imager.M1 microscope with a Plan-Neofluar objective (×40/0.75). The AxioVision software "Panorama" (Zeiss) generated a large composite image from the individual images representing the complete dentate gyrus. The actual thickness of the sections was 20 μm and two optical planes at the upper and lower slice surfaces were defined with the help of the AxioVision z-stack software (Zeiss). The stack was merged to one image with information gathered from all the in-focus labeled cells. Data are given as mean ± SEM, related to the complete dentate gyrus sub-/granular cell layer per slice.

The data from cell counting experiments were statistically analyzed with the non-parametric Kruskal–Wallis test. The Mann–Whitney *U* test was used as a post hoc test. Differences in the amounts of mRNA and protein levels were evaluated by one-way ANOVA. A *P* value of 0.05 or less was considered statistically significant.

Results

Cell proliferation

Ketamine treatment has no direct effect on cell proliferation in the rat hippocampus as demonstrated in Fig. 4. At decapitation point A (24 h after BrdU injection), there was no difference between saline (S/S) and ketamine (K/S)-treated groups. A 3-week withdrawal of ketamine, however, supported the survival of proliferated cells significantly (Fig. 4, decapitation point B). Risperidone (S/R) and haloperidol (S/H) showed the same effect. In saline-pretreated

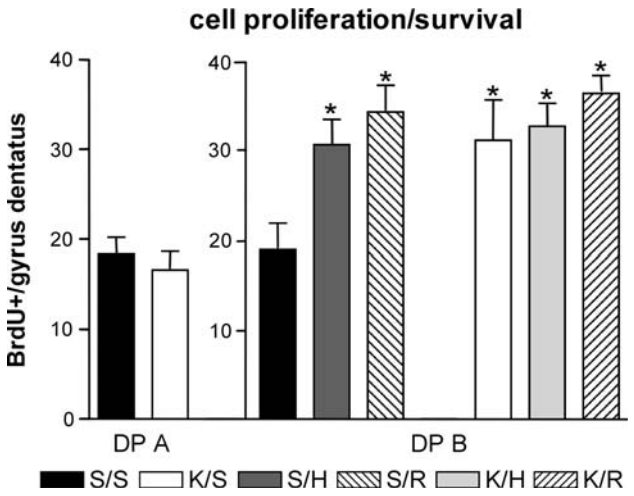

Fig. 4 Quantitative analysis of ongoing cell proliferation/survival in the subgranular cell layer of the lateral blade of the hippocampal dentate gyrus as measured in adult rats 24 h (*DP A*) and 3 weeks (*DP B*) after BrdU injection. The number of BrdU-immunoreactive nuclei of saline-treated (S/S) animals was compared with the respective numbers of all other groups. Additionally, the ketamine-treated group (K/S) was compared with the respective haloperidol (K/H) and risperidone (K/R) groups. Data are given as mean ± SEM, related to the complete dentate gyrus sub-/granular cell layer per slice. Differences from the control value (S/S) are indicated ($^*P < 0.05$) as analyzed by the non-parametric two-tailed *U* test (Mann and Whitney), *DP* decapitation point, *S* saline, *K* ketamine, *H* haloperidol, *R* risperidone

animals, application of both increased the total number of BrdU-labeled cells surviving for 3 weeks within the granule cell layer when compared with the S/S-injected control group significantly (Figs. 4, 5a vs. b, c). A discrete shift from the subgranular layer, the proper area of hippocampal neurogenesis, toward the middle part of the granule cell layer occurred independent of treatment (arrowheads in Fig. 5a, f). Significantly, more BrdU-labeled cells were found in the lateral than in the medial blade of the dentate gyrus (data not shown). In other hippocampal regions, e.g., CA1–CA3, BrdU-immunoreactive cells have been observed extremely seldom and we found no difference between the left and the right hippocampus (data not shown).

The neuroleptics had no effect on cell survival when applied in ketamine-pretreated animals. After 3 weeks of survival, the number of BrdU-labeled cells in ketamine/saline (K/S)-treated animals was comparable with that of ketamine/haloperidol (K/H)- and ketamine/risperidone (K/R)-treated rats (Figs. 4, 5d vs. e, f). The percentage of BrdU-labeled cells that were co-labeled with NeuN was enhanced after treatment with haloperidol (S/H, K/H) and risperidone (S/R, K/R) when compared with the S/S and K/S groups. In both of these groups, approximately 70% of BrdU-labeled cells had the morphology of granule cells, and were co-labeled with the neuronal marker NeuN (Fig. 5g). Most of them also expressed DCX (Fig. 5h). About 12% of BrdU-positive cells had the morphology of granule cell precursors and were labeled by the intermediate filament nestin, a marker for stem/progenitor cells (Fig. 5i). About 8% were co-immunolabeled with the astroglial marker GFAP (Fig. 5j). The remaining cells co-expressed Galac, a marker for oligodendroglia (~5%; Fig. 5k) or OX42, a microglia marker (~2%; Fig. 5l; overview in Table 2), or did not co-express any marker used. As a result of risperidone and haloperidol treatment, about 80% of BrdU-positive cells were co-labeled with NeuN. On the contrary, the number of cells expressing nestin (up to ~5%) or GFAP (up to ~5%) was reduced after this treatment.

mRNA expression

In the next setup of experiments, we evaluated the influence of ketamine, haloperidol, and risperidone on the mRNA expression of the neurotrophic factors NGF (Fig. 6a), BDNF (Fig. 6b) and NT-3 (Fig. 6c), known to play a role in cell proliferation and/or survival. Moreover, we measured the mRNA expression of their high-affinity receptors TrkA (Fig. 6d), TrkB (Fig. 6e) and TrkC (Fig. 6f) in the hippocampus. The mRNA level of BDNF (Fig. 6b) was the only one significantly ($P < 0.05$) affected by the pretreatment with ketamine (K/S). The mRNA levels of NGF, NT-3, TrkA, TrkB, and TrkC were unchanged. Haloperidol treatment (S/H) showed the tendency to depress the neurotrophins studied. However, the differences were not significant. Ketamine/haloperidol treatment had no effect on the expression of NGF, BDNF, NT-3, TrkB, and TrkC when compared with the saline/saline, the ketamine/saline or the haloperidol/saline groups. The TrkA expression (Fig. 6d) was enhanced after ketamine/haloperidol treatment compared to the ketamine/saline or haloperidol/saline groups ($P < 0.05$), whereas insignificantly changed in comparison to the saline/saline group. Risperidone/saline treatment showed a tendency to enhance the expression of NGF, TrkB, and TrkC, again without significance. The ketamine/risperidone treatment induced the same mRNA pattern with the exception of TrkA, the expression of which was enhanced ($P < 0.05$) in comparison to the groups treated with ketamine/saline or risperidone/saline.

The data suggested that the demonstrated treatment effects on cell survival must be mediated through activation of additional signaling pathways. To address this question, we measured the mRNA expression of VEGF (vascular endothelial growth factor, signaling protein involved in angiogenesis and cell proliferation in general) and MMP2 [matrix metallopeptidase 2 (gelatinase A), a proteolytic enzyme involved in cell proliferation, adhesion, and migration]. MMP2 mRNA expression was significantly

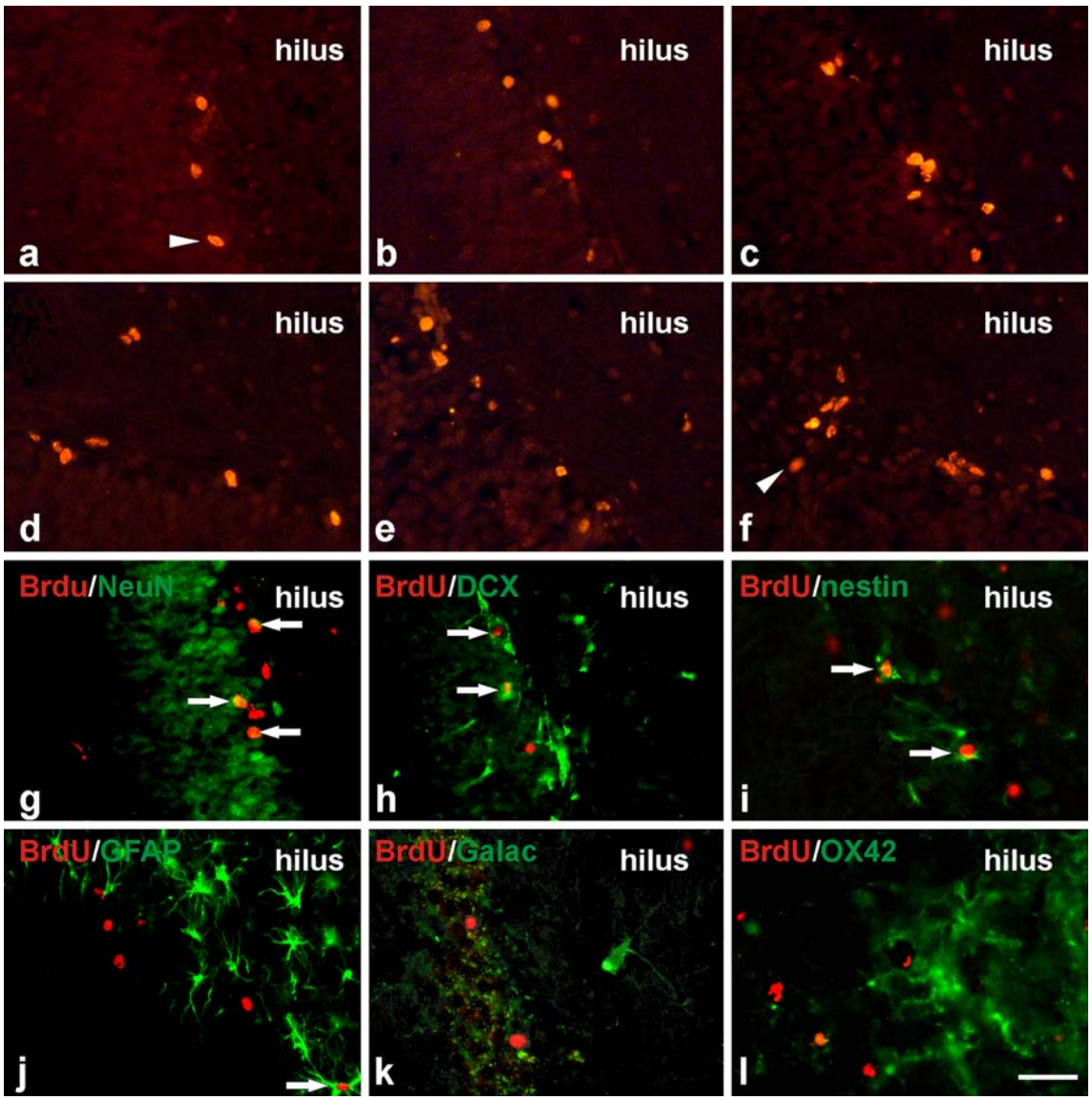

Fig. 5 After decapitation at point B (3 weeks after the cessation of BrdU treatment and 24 h after the last haloperidol/risperidone treatment), BrdU labeling of nuclei (*yellow dots*) of newly formed cells indicates a baseline mitotic activity in the hippocampus of sham-treated (S/S; **a**) animals. An increase in BrdU-incorporation is seen after haloperidol (S/H; **b**) and risperidone (S/R; **c**) as well as ketamine (K/S; **d**) treatment. In ketamine-pretreated animals, haloperidol (K/H; **e**) and risperidone (K/R; **f**) treatment did not alter the ketamine-induced cell proliferation/survival rate. The shift of BrdU-labeled cells from the subgranular layer toward the middle part of the granule cell layer has been started independent of treatment (*arrowheads* in **a**, **f**). Immunofluorescent double-labeling of BrdU-positive cells, demonstrated here with K/S animals, indicates co-expression with the neuronal marker NeuN (*arrows* in **g**), the neuronal precursor marker DCX (*arrows* in **h**) and with the stem/progenitor cell marker nestin (*arrows* in **i**). Co-labeling with the astroglial marker GFAP (*arrow* in **j**), the oligodendroglial marker Galac (**k**) or with the microglial marker OX42 (**l**) was scarce (*scale bar* 100 μm)

($P < 0.05$) enhanced in all experimental groups except the ketamine/saline group (Fig. 6g). The VEGF mRNA level was significantly ($P < 0.05$) higher in both risperidone groups (Fig. 6h). The changed expression of MMP2 and VEGF mRNA was adequately reflected at the respective protein levels (Fig. 7). Moreover, risperidone seemed to activate the protein expression of CREB as well as the phosphorylation of p38 MAP kinase (Fig. 7).

Cell death

Enhanced cell counts may be the result of intensified cell proliferation as well as a result of reduced cell death. Thus, we looked at the mRNA expression of the neuroprotective BCL-2 (B cell lymphoma 2, anti-apoptotic member of a family of anti- and pro-apoptotic proteins controlling the point of no return for cell survival), which was significantly

Table 2 Percentage (mean ± SEM) of BrdU-labeled cells in the granule layer of dentate gyrus those were co-labeled with the respective antibodies

Treatment	BrdU/NeuN	BrdU/DCX	BrdU/nestin	BrdU/GFAP	BrdU/Galac	BrdU/OX42
Saline/saline	70.4 ± 4.5	63.2 ± 4.4	11.8 ± 2.3	8.3 ± 2.1	4.7 ± 0.9	2.1 ± 1.1
Saline/ketamine	68.6 ± 6.2	63.8 ± 3.9	12.4 ± 3.0	9.1 ± 2.7	4.5 ± 2.3	3.2 ± 1.9
Saline/risperidone	79.7 ± 5.5*,#	62.4 ± 6.0	5.8 ± 2.7*,#	5.4 ± 2.2*,#	5.3 ± 3.6	2.0 ± 0.9
Saline/haloperidol	81.1 ± 3.8*,#	64.9 ± 5.4	4.9 ± 2.7*,#	5.1 ± 2.6*,#	4.9 ± 2.9	2.7 ± 1.3
Ketamine/risperidone	80.1 ± 5.9*,#	66.3 ± 3.6	6.1 ± 3.2*,#	4.7 ± 3.2*,#	5.3 ± 3.3	2.7 ± 1.3
Ketamine/haloperidol	78.9 ± 4.3*,#	62.8 ± 4.1	5.2 ± 2.4*,#	4.8 ± 3.2*,#	5.3 ± 3.5	2.2 ± 1.7

Data related to the complete dentate gyrus sub-/granular cell layer per slice. Significant differences to the respective control (S/S) values (*$P < 0.05$) and to the S/K values (#$P < 0.05$) are indicated

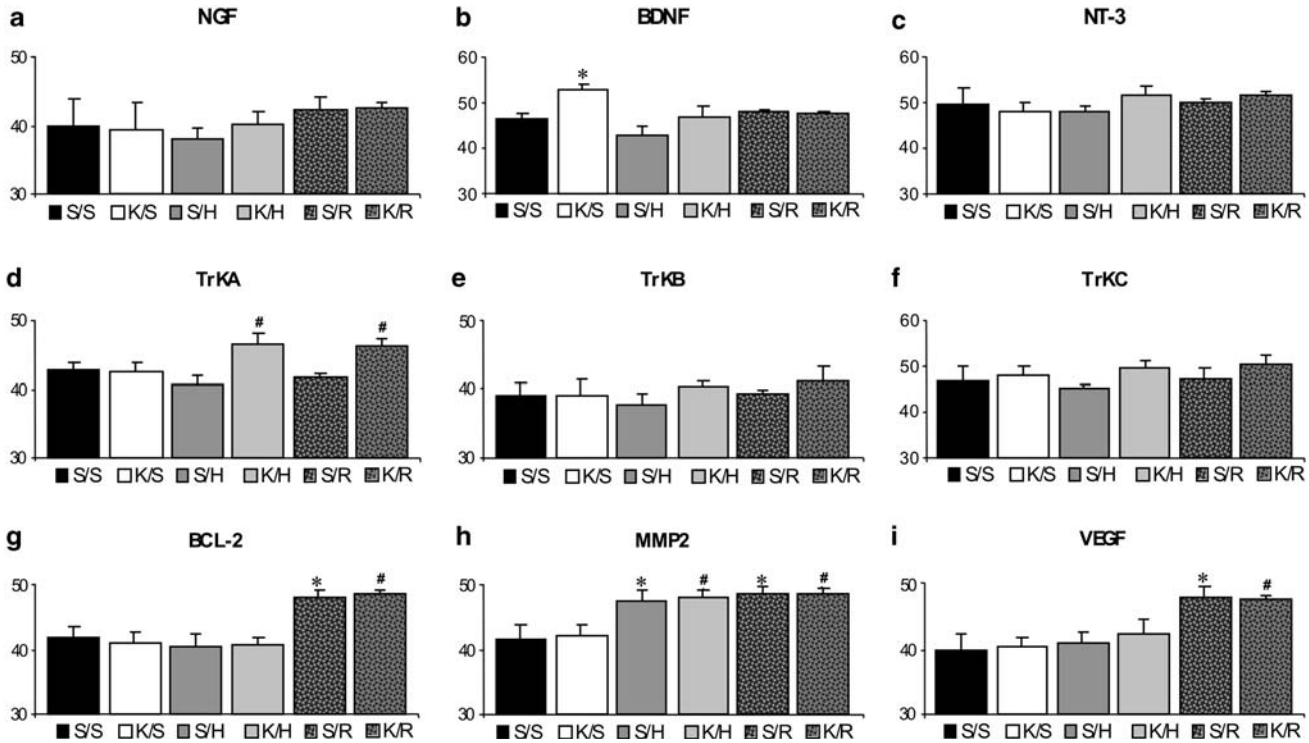

Fig. 6 Effects of haloperidol and risperidone on NGF, BDNF, NT-3, TrkA, TrkB, TrkC, BCL-2, MMP2, and VEGF mRNA levels in the hippocampus of sham (S/H or S/R vs. S/S) and ketamine (K/H or K/R vs. K/S)-pretreated rats. Mean ± SEM. Six animals per group were used (*$P < 0.05$ vs. S/S, #$P < 0.05$ vs. K/S); *S* saline, *K* ketamine, *H* haloperidol, *R* risperidone

($P < 0.05$) up-regulated in the risperidone groups (S/R, K/R; Fig. 6i).

TUNEL labeling revealed an enhanced cell death rate in the dentate gyrus of K/S-treated animals when compared with the S/S group. Risperidone and haloperidol did not influence the number of TUNEL-stained nuclei in the saline-pretreated (S/H, S/R) groups. The significantly enhanced cell death rate ($P < 0.005$) in ketamine-pretreated animals (K/H, K/R), however, was reduced, but this tendency was non-significant ($P < 0.1$; Fig. 8).

Discussion

In the present study, we demonstrated that risperidone and, to a lesser extent, haloperidol enhanced cell survival in the subgranular cell layer of the dentate gyrus of adult rat hippocampus. Thus, the known return of cognitive ability after risperidone and haloperidol treatment can be, at least in part, a result of their competence to restore normal neurogenesis and/or to enhance the survival of newly formed cell in case of diminished cell proliferation, though

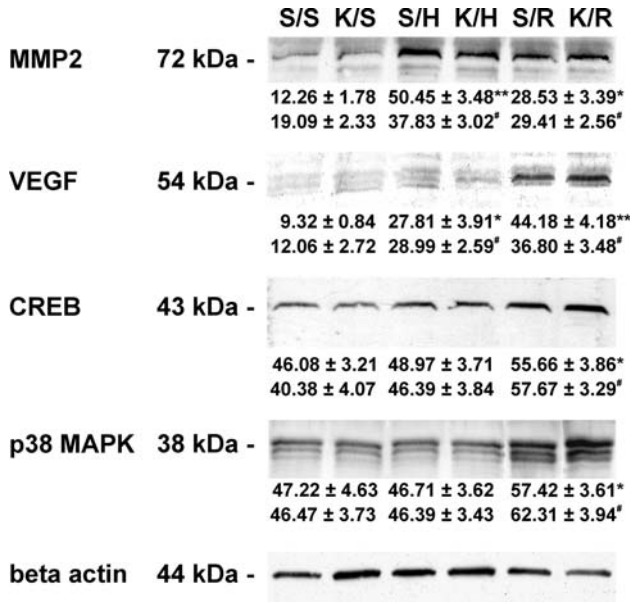

Fig. 7 Effect of haloperidol and risperidone on MMP2, VEGF, CREB and phosphorylated p38 MAP kinase protein levels in the hippocampus of sham (S/H or S/R vs. S/S, *upper line*) and ketamine (K/H or K/R vs. K/S, *lower line*)-pretreated rats. Immunoreactivity was quantified by densitometric analysis after normalizing the values with beta-actin in the same blot. Mean ± SEM. Six animals per group were used (*$P < 0.05$, **$P < 0.005$ vs. S/S, #$P < 0.05$ vs. K/S); *S* saline, *K* ketamine, *H* haloperidol, *R* risperidone

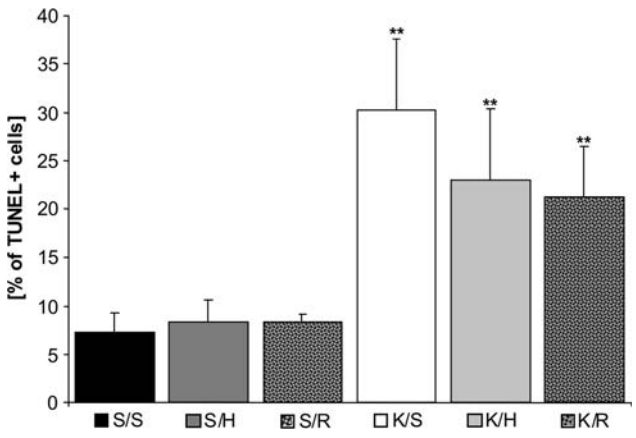

Fig. 8 Cell integrity of the dentate gyrus was assessed by TUNEL staining. In saline-treated animals, haloperidol (*S/H*) and risperidone (*S/R*) did not seem to contribute to cell death. The ketamine-induced cell death (*K/S*), however, tended to be reduced by haloperidol (*K/H*) and risperidone (*K/R*) treatment. Data are given as mean ± SEM, related to the complete dentate gyrus sub-/granular cell layer per slice (**$P < 0.005$ vs. S/S); *S* saline, *K* ketamine, *H* haloperidol, *R* risperidone

the production and survival of new cells does not guarantee functional recovery. Quite the opposite, cells with inappropriate connections or in aberrant locations could be detrimental for CNS function. In this study, a treatment schedule (duration, doses) was used, which was shown to normalize aspects of altered social behavior in ketamine-pretreated rats [3]. Although direct comparisons forbid themselves, our results are in line with the literature findings [32, 51]. That we found an effect of risperidone, others not [19], might be a problem of the quite different experimental settings. Thus, we use the i.p. application regime, whereas in [19] risperidone was applied via drinking water. In case of haloperidol, on the other hand, the effect on granule cell proliferation in the gerbil hippocampus [11] could be found only after application of a dosage which was more than 25 times higher than the dose used in our experiments.

As RT-PCR revealed that the treatment effects of risperidone and haloperidol seemed to be essentially mediated through activation of factors apart from the classic neurotrophins NGF, BDNF, and NT3. In our experiments, these factors were widely unaffected by risperidone and haloperidol except the increase of mRNA expression of TrkA after pretreatment. Other groups have even shown a reduction of NGF or BDNF [2, 9]. The up-regulation of TrkA without stimulation of NGF might be one reason for the fact that we could not find an additive proliferative effect of ketamine withdrawal and haloperidol/risperidone. It is known that overexpressing TrkA neurons become dependent on the respective neurotrophin for survival [13].

One candidate of interest was VEGF, which was initially characterized for its role in angiogenesis, but also exerts direct mitogenic effects on neural progenitors in vitro [52]. In our paradigm, risperidone activated VEGF, identifying VEGF as one possible mediator of its cytogenic action. The signaling pathway by which risperidone might induce VEGF expression could involve the cAMP response element binding protein (CREB). Risperidone treatment increases the phosphorylation of CREB in cultured hippocampal neurons [52], and the VEGF promoter contains a cAMP response element that is induced by receptor-coupled pathways that activate CREB [23]. Afterward, VEGF regulates its downstream effectors such as PI3K/Akt and MEK/ERK1/2. The significant increase of ERK1/2 phosphorylation [53] as well as the cell-protective potency shown here can also be explained by this mechanism. Loss of ERK1/2 phosphorylation [17] or a down-regulation of ERK1/2 activity [37] correlated significantly with increased cell death. Haloperidol had no effect on VEGF expression, which is in line with findings that haloperidol did not induce ERK1/2 activity [1].

Furthermore, we found a risperidone- and haloperidol-mediated induction of MMP2. In the case of risperidone, this effect could be a result of its effect on VEGF expression, as VEGF induces MMP-2 expression via JNK [24]. A direct effect must be assumed for haloperidol. MMP2, on the other hand, unmasks VEGF by processing the VEGF binding proteins HARP (heparin affine regulatory peptide)

and CTGF (connective tissue growth factor) [12], thus further enhancing the cell-protective potency. Additionally, we found a risperidone-mediated induction of BCL-2. That is in line with findings that temporal cortices of schizophrenics treated with antipsychotic drugs show nearly double the BCL-2 expression of untreated patients [25]. In accordance with this group, we did not detect a haloperidol effect on BCL-2 expression in rats [26, 27]. Kuhn and coworkers [33] demonstrated an increased generation of granule cells in adult BCL-2-overexpressing mice. Thus, the cell-protective potency of risperidone and haloperidol, including their revealed differences (haloperidol is less effective) might result from their different effects on VEGF/MMP2 and BCL-2 expression.

A further possibility by which risperidone and haloperidol may influence cell proliferation/survival is their effect on nitric oxide (NO). From NO, it is known that it regulates adult neurogenesis; this, however, in dependence on the origin of NO. NO produced by neuronal NOS is a negative regulator of neurogenesis [41], whereas NO produced by endothelial NOS seems to be necessary for brain plasticity [8]. It enhanced the production of BDNF, which, in turn, reduces the activity of neuronal NOS, thus inducing neurogenesis. Risperidone and haloperidol, on the other hand, inhibited the production of NO, whereby the inhibitory effect of risperidone was much stronger than those of haloperidol [30].

Up to what extent ketamine-withdrawal affects proliferation of neuronal stem cells directly is unverifiable by the protocol used. However, the induction of hippocampal neurogenesis by drug withdrawal is a known phenomenon. In a model of alcohol dependence, it was shown that adult neurogenesis is inhibited during dependence with a pronounced increase in new hippocampal neuron formation after abstinence [39]. And withdrawal of growth factors promotes proliferation of neurons and glia by repressing intrinsic programs of stem cell differentiation [40]. Obviously, temporal aspects may play a crucial role in processes related to neurogenesis/cell survival. Phencyclidine (PCP), like ketamine an antagonist at the NMDA receptor, induces transient disturbances of cell proliferation within dentate gyrus which returned to control levels within 1 week [35]. Applied on the experiments concerning the neurogenetic impact of NMDA receptor antagonists, it is easy to speculate that depending on the duration of treatment and of withdrawal before exploration, investigators could see either inhibition, increase or no change. Moreover, differences in the mode of action of the NMDA antagonists used are of importance. For example, ketamine shows very similar affinity at the NMDA receptor and D2 binding sites, with a lower affinity for the NMDA and 5-HT2 sites, while PCP shows similar affinity for the NMDA and 5-HT2, with a lower affinity for the D2 sites [29].

Ketamine withdrawal has no effect per se on the expression of VEGF, MMP2 and BCL-2. It seemed to achieve the demonstrated cell proliferative/protective effect by the activation of BDNF. This inductive effect has also been found by other groups [7, 16]. The cell proliferation/survival induced by ketamine withdrawal was not affected by haloperidol and risperidone. The expression levels of BDNF in the K/R and K/H groups were comparable to these of the S/S, S/R, and S/H groups, whereas the K/S group reached a significantly higher level. These differences, however, were not reflected at the level of cell proliferation. Possibly it was overlaid by the VEGF/MMP2 effects of risperidone and haloperidol.

Interestingly, risperidone, but not haloperidol, effectively counteracts several ketamine- and PCP-induced behavioral alterations in animal models [3, 18]. We speculated that this might be a result of their different modes of action on multiple neurotransmitter receptors, especially dopamine D2 and 5-HT2A receptors [3]. In the present experiments, we found that ketamine treatment had no acute effect on neurogenesis, whereas its withdrawal enhanced the survival of newly generated cells and that neither risperidone nor haloperidol did influence this effect. Both neuroleptics, on the other hand, promoted survival of newly generated cells, which supports, at least in part, the "neurogenetic" hypothesis of schizophrenia. Once again, we have to underline that a more of neurons does not lead automatically to the improvement of brain function. Nevertheless, promoting survival may be one way in which risperidone and haloperidol might realize the improvement of some behavioral aspects in animal models of schizophrenia; an idea which is discussed for antidepressants quite a long time [36].

Schizophrenia is a developmental disease. In the ketamine paradigm, however, young-adult animals were used. Thus, further experiments should investigate the neurogenetic effect of neuroleptics in developmental models of schizophrenia, e.g., postnatal lesion of the ventral hippocampus or prenatal vitamin D deficiency.

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