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New horizons in schizophrenia treatment: autophagy protection is coupled with behavioral improvements in a mouse model of schizophrenia

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Abbreviations: *Adnp*, activity-dependent neuroprotective protein (mouse); *ADNP*, activity-dependent neuroprotector homeobox (human); *Adnp2* (mouse), *ADNP2* (human), ADNP homeobox 2; *Becn1* (mouse), BECN1 (human), Beclin 1, autophagy-related; CLZ, clozapine; *Hprt*/*Hprt1*, hypoxanthine phosphoribosyl transferase; *Map1lc3b* (mouse), *MAP1LC3B* (human), microtubule-associated protein 1 light chain 3 β; *Map6* (mouse), *MAP6* (human), microtubule-associated protein 6.

Autophagy plays a key role in the pathophysiology of schizophrenia as manifested by a 40% decrease in *BECN1*/*Beclin 1* mRNA in postmortem hippocampal tissues relative to controls. This decrease was coupled with the deregulation of the essential ADNP (activity-dependent neuroprotector homeobox), a binding partner of MAP1LC3B/LC3B (microtubule-associated protein 1 light chain 3 β) another major constituent of autophagy. The drug candidate NAP (davunetide), a peptide fragment from ADNP, enhanced the ADNP-LC3B interaction. Parallel genetic studies have linked allelic variation in the gene encoding MAP6/STOP (microtubule-associated protein 6) to schizophrenia, along with altered MAP6/STOP protein expression in the schizophrenic brain and schizophrenic-like behaviors in *Map6*-deficient mice. In this study, for the first time, we reveal significant decreases in hippocampal *Becn1* mRNA and reversal by NAP but not by the antipsychotic clozapine (CLZ) in *Map6*-deficient ($Map6^{+/-}$) mice. Normalization of *Becn1* expression by NAP was coupled with behavioral protection against hyperlocomotion and cognitive deficits measured in the object recognition test. CLZ reduced hyperlocomotion below control levels and did not significantly affect object recognition. The combination of CLZ and NAP resulted in normalized outcome behaviors. Phase II clinical studies have shown NAP-dependent augmentation of functional activities of daily living coupled with brain protection. The current studies provide a new mechanistic pathway and a novel avenue for drug development.

Introduction

Schizophrenia affects approximately 1% of the adult population.[1] Schizophrenia patients have 3 major symptoms: positive symptoms (including delusions and hallucinations), negative symptoms (including anhedonia, the inability to begin and maintain planned activities) and cognitive symptoms (including memory deficits, difficulties in focusing, and short attention span).[2] The core treatment for schizophrenia is antipsychotic medications,[3] which do not address general functioning, thus maintaining cognitive impairment associated with schizophrenia as an unmet global healthcare problem.[4,5]

Autophagy is a cellular process that preserves the balance between the synthesis, degradation, and recycling of cellular components. Autophagy is essential for neuronal survival and function and was recently suggested to play a key role in the pathophysiology of schizophrenia.[6] Several proteins control the autophagy pathway, including BECN1 and MAP1LC3 (LC3 henceforth in the text). BECN1, triggering the formation of autophagosomes via membrane recruitment,[7] exhibits reduced expression in the hippocampus of postmortem schizophrenia subjects but not in peripheral lymphocytes.[6] LC3, serving as a marker for autophagy, is cleaved at the C terminus to become LC3-I and is transformed by conjugation to

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phosphatidylethanolamine (PE) to produce LC3-II. LC3-II connects to the phagophore membrane.[8] ADNP, an essential protein for brain formation,[9,10] interacts with LC3. This interaction is increased in the presence of the short ADNP peptide and the therapeutic drug candidate NAP.[6] In contrast to BECN1, the expression of *Adnp* and its family member *Adnp2*,[11-14] which is deregulated in postmortem hippocampal samples from schizophrenia subjects,[15] showed significantly increased expression in lymphocytes from related patients. Such augmentation is similar to increases in the antiapoptotic protein BCL2 (B-cell CLL/lymphoma 2), which interacts with BECN1. The increase in ADNP was associated with the initial stages of the disease, possibly reflecting a compensatory effect. The increase in ADNP2 might be a consequence of neuroleptic treatment, as observed in rats subjected to CLZ treatment.[6]

Genome-wide association analysis encompassing thousands of schizophrenia patients has identified risk loci for schizophrenia. Identified genes included those encoding proteins associated with calcium, glutamate, and N-methyl-D-aspartate/NMDA signaling in addition to protein degradation, cytoskeleton, and synapse assembly.[16,17] In this respect, ADNP may affect schizophrenia, in part through interaction with microtubules.[18,19] *Adnp* haploinsufficiency results in cognitive and social deficits, paralleled by microtubule-associated pathology; treatment with the ADNP snippet NAP (a microtubule-stabilizer) provides partial protection from these effects.[20-22] and pipeline drug candidates.[23,24] *Adnp* haploinsufficiency is also associated with decreased hippocampal *Becn1* expression.[6] It remains to be observed if NAP and/or pipeline products can protect against BECN1 deficiency.

Schizophrenia is now conceptualized as a syndrome with distinguishable symptoms, such as psychosis, cognitive dysfunction, and negative symptoms. This description has been extended to animals, thus allowing for the possibility of identifying animal models of schizophrenia and/or the aforementioned manifestations of the disorder. *Map6*-deficient mice fulfill the criteria of the above model. Indeed, *Map6*-deficient mice exhibit severe behavioral disorders that can be linked to positive and negative symptoms in addition to cognitive dysfunction.[25-32] Additionally, *Map6*-null mice fulfill the 3 criteria: construct validity, face validity and predictive validity, all required for a valid animal model of psychiatric disorder. Concerning construct validity criteria, as in humans, *map6*-null mice exhibit abnormal neuronal connectivity linked to synaptic and neurotransmission defects (hypoglutamatergic neurotransmission and dopamine hyperreactivity).[32-37] With respect to face validity criteria, *map6*-null mice exhibit severe and numerous behavioral defects related to psychiatric symptoms, including hyperactivity, anxiety, social withdrawal, increased resignation, and working memory defects.[25,28,29,32,38-40] Regarding predictive criteria, these mice demonstrate several behavioral and biological deficits that are sensitive to typical and atypical antipsychotics, as well as antidepressants.[32,38,39,41]

Interestingly, the taxol-like microtubule stabilizer epothilone D ameliorates synaptic impairment and schizophrenic-like behavior in *Map6*-deficient mice.[31,42] Our recent study[43] showed that either NAP or CLZ treatment significantly reduced the $Map6^{+/-}$ mouse positive symptoms (hyperactivity) in the open field. However, as opposed to CLZ, NAP treatment significantly ameliorated cognitive deficits in the *Map6*-deficient mouse model. Phase II clinical studies have shown NAP-dependent improvement of functional activities of daily living coupled to brain protection.[44,45]

We now ask the following questions: 1) Are autophagy-associated proteins deregulated in the *Map6*-deficient mouse brain, mimicking the human schizophrenia brain? 2) Will the treatment of $Map6^{+/-}$ mice with NAP reverse MAP6-related deficiencies, including normalization of the expression of autophagy-related proteins? 3) Will CLZ mimic the NAP effect or will a combination be beneficial?

We postulate that a combination of NAP and CLZ may provide not only inhibition of psychosis (CLZ) but also functional strengthening of activities of daily living and rational cognitive behavior (NAP).

Results

$Map6^{+/-}$ mice, similar to human schizophrenia subjects, show BECN1 deficiency, which is ameliorated by NAP + CLZ combination treatment

Our recent studies show a 40% decrease in *BECN1* mRNA in the hippocampus of postmortem schizophrenic subjects compared to age-matched controls.[6] It was therefore of interest to investigate if a similar reduction occurs in the *Map6*-deficient mouse model, thus validating the model for future preclinical testing. Our immunohistochemical studies investigating the hippocampal area showed a statistically significant decrease in the number of cells stained with BECN1 antibody in the $Map6^{+/-}$ mice (~3-fold; $^{*}P < 0.05$) compared to the $Map6^{+/+}$ mice (**Fig. 1A**, $Map6^{+/+}$ staining; B, $Map6^{+/-}$ staining and C, graphical comparison). This result was verified at the RNA expression level (**Fig. 2**). Hippocampal RNA expression level was measured by quantitative real-time PCR. Our results showed that *Becn1* RNA levels were significantly lower in the $Map6^{+/-}$ mice compared to the $Map6^{+/+}$ mice (40% reduction, **Fig. 2A**, $^{***}P < 0.001$), mimicking the reduction in the human brain.[6] CLZ treatment of $Map6^{+/-}$ mice did not affect *Becn1* RNA levels (**Fig. 2B**, $P = 0.347$). In contrast, NAP treatment significantly increased *Becn1* RNA (**Fig. 2C**, $^{*}P < 0.05$) in the $Map6^{+/+}$ mouse level (**Fig 2A**). This increase in *Becn1* transcript expression in the presence of NAP was maintained in combination with CLZ treatment (**Fig. 2D**, $^{*}P < 0.05$).

The hippocampal expression of the NAP parent protein ADNP and the related protein ADNP2 were downregulated in *Map6*-deficient mice (for either ADNP, or ADNP2, $^{***}P < 0.001$). CLZ treatment was associated with a further apparent reduction of *Adnp* expression (**Fig 2B**, $P = 0.07$), which may be indicative of unwanted behavioral side effects (decreased cognitive performance), as measured previously in $Adnp^{+/-}$ mice.[23] Neither NAP nor combination treatment affected *Adnp* RNA expression. However, NAP treatment resulted in a slight reduction in *Adnp2* levels, which was not apparent in combination with CLZ (**Fig. 2C and D**, *$P < 0.05$). Interestingly, previous results have shown a deregulation of ADNP and ADNP2 in schizophrenia with *ADNP2* RNA levels increasing in the hippocampus of the postmortem brains in parallel with disease duration.[15] Our current results suggest that NAP treatment may protect against this deregulation.

These results prompted further experiments to evaluate and correlate behavioral outcomes to protein expression with an emphasis on autophagy.

Map6$^{+/-}$ mice hyperactivity in the open field is normalized by NAP + CLZ combination treatment

As previously described,[43] *Map6*$^{+/-}$ mice suffer from hyperactivity compared to *Map6*$^{+/+}$ mice. This hyperactive behavior in *Map6*$^{+/-}$ mice has previously been linked to positive symptoms in human schizophrenia.[32,35,37] Our current results confirmed this finding, showing that *Map6*$^{+/-}$ mice (n = 13) were hyperactive, traveling approximately twice the distance over the same time period compared to the control *Map6*$^{+/+}$ mice (**Fig. 3A**, ***$P < 0.001$).

Three wk of daily treatment of the *Map6*$^{+/-}$ mice with either CLZ compared to subcutaneous (s.c.) vehicle control (**Fig. 3B**) or NAP compared to intranasal vehicle control (DD[43,46]) (**Fig. 3C**) significantly reduced hyperactivity in the open field test. In the presence of CLZ, hyperactivity was reduced by ~5-fold (***$P < 0.001$), and with NAP, hyperactivity was reduced by ~2-fold (**$P < 0.01$). CLZ treatment dramatically reduced activity to levels even lower than those observed in the *Map6*$^{+/+}$ mice (possibly indicating lethargy/somnolence from drug treatment as observed in patients).[47] The *Map6*$^{+/-}$ mice treated with the combination of NAP and CLZ showed activity that matched the behavior of the normal naïve mice (**Fig. 3A** compared to 3D, **$P < 0.01$, placebo treatment compared to combination). Together, our results showed that CLZ, NAP and combination treatment reduced *Map6*$^{+/-}$-deficient-related hyperactivity compared to the appropriate vehicle controls (**Fig. 3B to D**).

The schizophrenic-like preference for familiar rather than novel objects typically exhibited by *Map6*$^{+/-}$ mice is normalized by NAP + CLZ combination treatment

The object recognition and discrimination test, based on visual discrimination between 2 different objects, measures object-related memory, which is deficient in schizophrenia.[48] Although one may measure several schizophrenia-related behavioral deficits, as elegantly reviewed,[48] we concentrated on object recognition, as our previous data showed an association of *Map6*-deficiency with reduced performance in the object recognition test.[43] Here, we extended these studies; *Map6*$^{+/-}$ mice were not able to discriminate the familiar object from the novel object and preferred the familiar object, an observation that was in contrast to the control *Map6*$^{+/+}$ mice (**Fig. 4A**, discrimination index −0.35 vs. 0.24; **$P < 0.01$ for genotype effect). CLZ treatment did not reach significance ($P = 0.47$, **Fig. 4B**). In contrast, the NAP-treated *Map6*$^{+/-}$ mice (**Fig. 4C**) and the NAP+CLZ–treated *Map6*$^{+/-}$ mice (**Fig. 4D**) behaved like the *Map6*$^{+/+}$ naïve mice, while significantly differing from the respective vehicle-treated *Map6*$^{+/-}$ mice (**$P < 0.01$ for either of the groups, **Fig. 4C and D**).

Interestingly, the NAP-treated *Map6*$^{+/-}$ mice exhibited an even greater discrimination index compared to the *Map6*$^{+/+}$ mice (**Fig. 4A** versus 4C). However, with the combination treatment of NAP + CLZ, the mice showed almost identical scores compared to those of the *Map6*$^{+/+}$ mice (normal naïve mice).

CLZ toxicity and NAP protection

Published literature links CLZ to neuronal cell death in vitro,[49] which may be prevented by NAP treatment.[50] Using the neuronal human SH-SY5Y neuroblastoma cell line, we set out to verify 1) whether CLZ indeed harms neuronal-like cellular viability and 2) whether NAP reduces cell death caused by CLZ

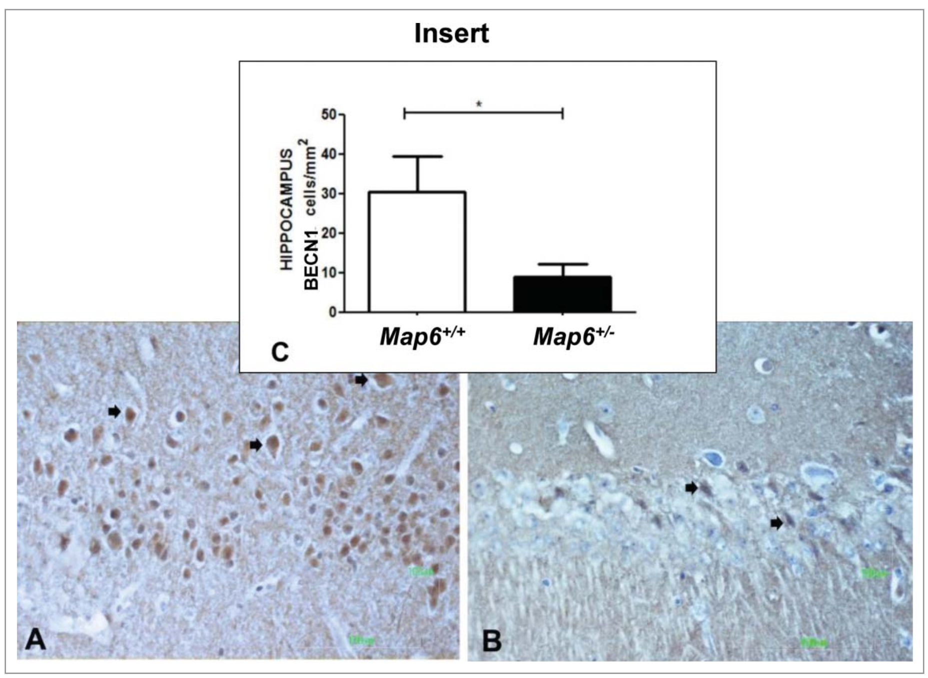

Figure 1. *Map6*$^{+/-}$ mice, like human schizophrenia subjects, show hippocampal *Becn1* deficiency. Experiments are detailed in Materials and Methods. Panels (**A–C**) compare naïve *Map6*$^{+/+}$ to naïve *Map6*$^{+/-}$ mice. A representative stained section is shown in (**A**) for *Map6*$^{+/+}$ and a representative stained section is shown in (**B**) for *Map6*$^{+/-}$ mice (arrows denote stained cells). Panel (**C**, insert) shows the comparison between the stained cell number/mm_2 in (**A**) vs. (**B**), showing approximately 3-fold reduction in *Map6*$^{+/-}$ mice as compared to controls (Student *t* test, *$P < 0.05$).

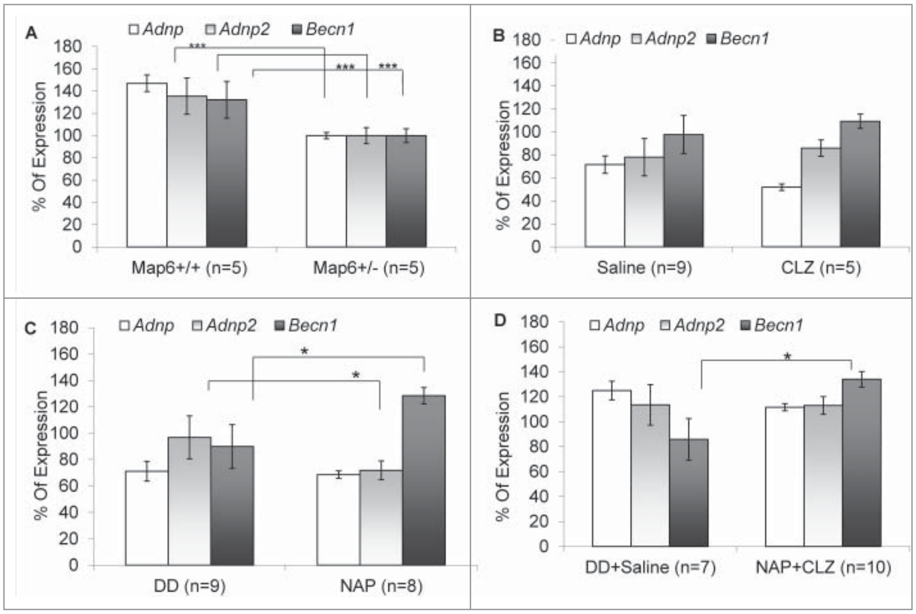

Figure 2. *Becn1* expression-deficiency in *Map6*$^{+/-}$ mice is ameliorated by NAP + CLZ combination treatment. Quantitative real-time PCR was performed for *Adnp* (white bars), *Adnp2* (gray bars) and *Becn1* (black bars). Results are depicted in graphs of percentage of expression of the naïve *Map6*$^{+/-}$ mice. Each transcript was compared to itself in *Map6*$^{+/-}$ mice vs. *Map6*$_{+/+}$ mice (**A**) or the comparative treatment with the relevant vehicle, all in the *Map6*$^{+/-}$ mice (Saline vs. CLZ+/- (i.e. saline-treated *Map6*$^{+/-}$ mice or CLZ treated *Map6*$^{+/-}$ respectively), **B**; DD vs. NAP+/- (i.e., DD-treated *Map6*$^{+/-}$ mice or NAP treated *Map6*$^{+/-}$ respectively), **C** and DD+Saline vs. NAP+CLZ+/-, **D**) using the Student *t* test. Panel **A** compares *Map6*$^{+/-}$ mice to *Map6*$_{+/+}$ mice. A significant ~40% decrease was observed for all tested genes in the *Map6*$^{+/-}$ mice (***$P < 0.001$). Panel (**B**) shows no effect for CLZ treatment when compared to saline injection, while panel (**C**) shows a significant effect of NAP on *Becn1* expression and *Adnp2* expression (with NAP normalizing *Becn1* expression) in comparison to vehicle only, (*$P < 0.05$). Panel (**D**) shows that the combination of CLZ+NAP normalized *Becn1* expression (*$P < 0.05$).

treatment. Human SH-SY5Y neuroblastoma cells were exposed to NAP (10^{-15} M to 10^{-11} M) for 24 h at 37°C either in the absence or in the presence of 20 μg/ml CLZ.[49] The measurement of metabolic activity/viability was performed by a cell proliferation assay (MTS). NAP treatment at concentrations $\geq 10^{-15}$ M prevented CLZ-associated reduced viability in the SH-SY5Y cell line (one-way ANOVA, F(3) = 103.618, $P < 0.001$ for treatment; the Fisher Least Significant Difference (LSD) post-hoc analysis in comparison to the CLZ only condition (see Materials and Methods section) revealed protection by NAP concentration of 10^{-15} M to 10^{-13} M: ***$P < 0.001$), while NAP alone did not affect cell viability, under these experimental conditions (**Fig. 5A and B**, respectively).

Discussion

Standard of care in schizophrenia may give a suitable treatment against the positive symptoms but not against the negative symptoms. As a result, a large number of patients fail to lead a normal life. Therefore, it is extremely important to provide treatments addressing all aspects of the disease, encompassing both positive as well as negative symptoms and cognitive dysfunctions. A reliable animal model, imperfect as it may be, enables the prediction of treatment efficiency via mouse behavior and the potential elucidation of disease mechanisms.

CLZ, used in clinical practice, has an impact on the positive symptoms of schizophrenia. At the same time, however, cognitive and daily functional activities of schizophrenia patients remain an unmet medical need. Our results confirmed previous studies showing a beneficial effect of CLZ on the positive symptoms (hyperactivity, although with reduction below normal control levels) with limited, insignificant effects on object recognition.[43] NAP treatment showed a significant reduction in hyperactivity, albeit less effective than CLZ. Furthermore, NAP showed a significant impact on the "cognitive" symptoms of the mice (differentiation between novel and familiar objects). The combination treatment resulted in the normalization of the impact on the positive symptoms (hyperactivity) in the open field and imparted a significant increase in cognitive abilities in the object recognition test relative to the CLZ treatment but not over the NAP treatment alone.

The combination of NAP in addition to traditional treatment might better affect the clinical outcome relative to traditional treatment alone. Our results in the SH-SY5Y cells led us to conclude that CLZ treatment, albeit at concentrations 30- to 60-fold higher than the optimal dose, reduced mitochondrial function, and cellular viability. NAP treatment prevented CLZ-associated decreases in cellular viability. The most recent studies have shown that CLZ reduces neuronal cell viability through the blockade of autolysosome formation,[51] and our studies show that NAP increases the autophagic process.[6,52]

Real-time PCR RNA expression analysis revealed a significant reduction in *Becn1* transcripts in the hippocampus of the *Map6*$^{+/-}$ schizophrenia-like mouse model relative to the *Map6*$^{+/+}$ mice. Whereas all placebo-treated groups demonstrated the same reduced expression level as the *Map6*$^{+/-}$ naïve mice, the mice that were treated with NAP alone, or with NAP in combination with CLZ demonstrated higher (similar to *Map6*$^{+/+}$ naïve) levels of *Becn1* transcripts. Perhaps through autophagy or other key related processes, BECN1 might play a significant role in the *Map6*$^{+/-}$ model of schizophrenia. The decrease in *Becn1* in the hippocampus of the *Map6*$^{+/-}$ mouse is in line with our recent detection of reduced *BECN1* expression in the postmortem human hippocampus from schizophrenia subjects compared to that in the controls.[6] These similarities between findings in mouse and human strengthen the validity of the *Map6*$^{+/-}$ model as a platform for further drug testing, with NAP as a potential lead compound.

Interestingly, the loss of MAP6 protein in *map6*-null mice impairs peripheral olfactory neurogenesis.[53] Thus, *Map6*-deficient olfactory neurons showed presynaptic swellings with autophagic-like structures. In olfactory and lvomeronasal epithelia, there was an increase in neuronal turnover, as shown by an increase in the number of proliferating, apoptotic, and immature cells with no changes in the number of mature neurons. Similar alterations in peripheral olfactory neurogenesis have also been described in schizophrenia patients.[54] Furthermore, regeneration resulted in the abnormal organization of olfactory terminals within the olfactory glomeruli in *map6*-null mice.[53] These complementary studies, compared to ours, suggest regional variations in neuronal regulation with abnormalities in the autophagic and apoptotic mechanisms resulting from MAP6 deficiencies, which may mimic the clinical/pathological manifestation in schizophrenia.

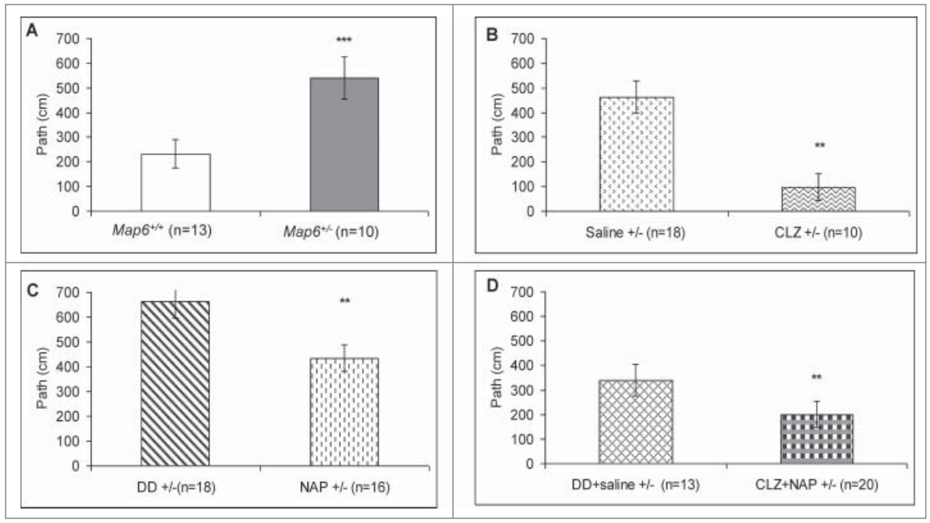

Figure 3. *Map6*$_{+/-}$ mice suffer from hyperactivity in the open field which is normalized by NAP + CLZ combination treatment. The results are shown as the mean of the path in cm per animal in the 3-min time period. *Map6*$_{+/+}$ (Sv129) controls exhibited a significant difference from their and *Map6*$_{+/-}$ (heterozygous) littermates (**A**,***$P < 0.001$, Student *t* test). Panel (**B**) compares CLZ and saline (vehicle)-treated *Map6*$_{+/-}$ mice. CLZ significantly reduced hyperactivity in the *Map6*$_{+/-}$ mice (***$P < 0.001$, Student *t* test). Panel (**C**) compares intranasal NAP and intranasal DD (vehicle)-treated *Map6*$_{+/-}$ mice. NAP significantly reduced hyperactivity in the *Map6*$^{+/-}$ mice (**$P < 0.01$, Student *t* test). Panel (**D**) compares combination treatment of NAP and CLZ to the appropriate vehicle control showing significant protection against hyperactivity (**$P < 0.01$, Student *t* test). In (**D**), treatment with DD+saline significantly decreased activity, possibility associated with the repeated handling in comparison to the *Map6*$^{+/-}$ mice (from 3A, $P < 0.001$, Student *t* test).

Our immunohistochemical data expanded upon the biochemical analyses, showing a marked decrease in the number of cells expressing BECN1 in the hippocampus of the naïve *Map6*$^{+/-}$ mice compared to that in the naïve *Map6*$^{+/+}$ mice. The autophagic process is in a crossroad between cell viability and apoptosis; we hypothesize that the deregulation of autophagy induces cell death. The putative effects on autophagy are consistent with NAP protection against apoptosis, which includes protection against caspase activation, as observed in our previous studies[55,50] and mitochondrial CYCS/cytochrome C release[56] in association with the microtubule-cytoskeleton protection.

Interestingly, previous findings show an inhibitory effect of schizophrenia drugs, including CLZ, on the autophagic process[51] and the inhibition of autophagy by the recruitment of BECN1 to the microtubules.[57] Here, we showed that NAP alone and in combination with CLZ increased *Becn1* expression. We suggest that to effectively treat schizophrenia, it is necessary for the cell to have adequate cytoskeleton, *BECN1* expression and apoptosis to provide for an efficient process of autophagy and/or other *BECN1*-related processes. Furthermore, the schizophrenia-related deregulation of *ADNP* and *ADNP2* expression that has previously been observed in the human hippocampus was recapitulated here to a certain degree in the *Map6*$^{+/-}$ mouse model.[15] The association of ADNP and its fragment peptide NAP with the microtubule cytoskeleton[58] and the autophagy pathway[6,52] paves the path to replacement therapy.

Recent clinical studies have shown significant efficacy for NAP treatment on the UCSD Performance-based Skills Assessment of functional activity.[44] Further studies show NAP protection of N-acetylaspartate and choline levels in dorsolateral prefrontal cortex in patients with schizophrenia, suggesting neuroprotection *in vivo*.[45] These results support the preclinical prediction and suggest future larger clinical testing, combination treatments and further investigation of pipeline products, leading to science-based translational medicine.

Methods and Materials

Animals and treatment

Five-mo-old *Map6*$^{+/-}$ and *Map6*$^{+/+}$ male mice were received at Tel Aviv University under a material transfer agreement from Annie Andrieux and Didier Job (INSERM). The mice were housed under controlled environmental conditions with a 12 h light and 12 h dark cycle. All experiments were conducted according to the guidelines for care and use of laboratory animals at Tel-Aviv University, under governmental permission.

We conducted an in vivo study with ~100 *Map6*$^{/-}$ mice and 10 *Map6*$^{+/+}$ mice. The experiment included 2 groups of naïve-

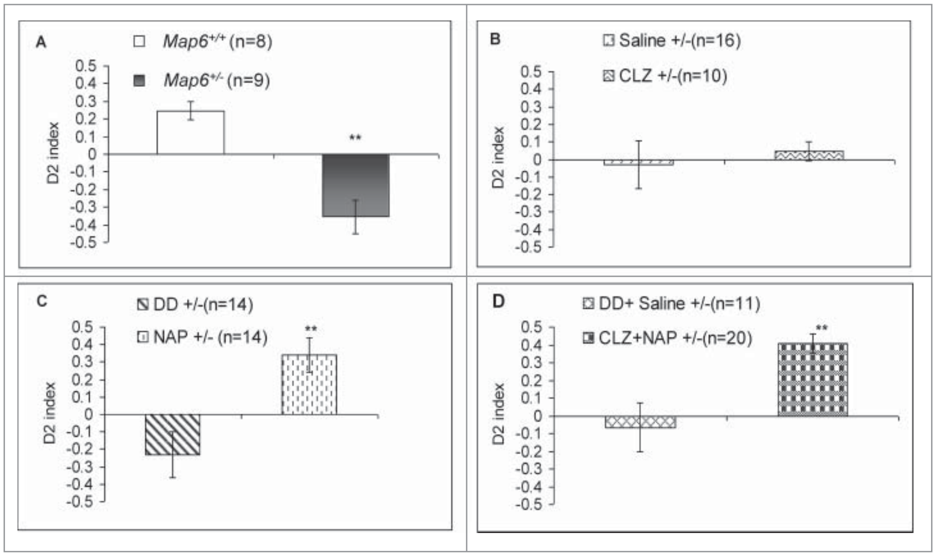

Figure 4. The *Map6*$^{+/-}$ mice schizophrenic-like preference to familiar rather than novel objects is normalized by NAP + CLZ combination treatment. *Map6*$_{+/-}$ mice showed significant deficits in recognizing the novel object and seemed to significantly prefer the familiar object in comparison to control *Map6*$_{+/+}$ mice (**A**,$^{**}P < 0.01$, Student *t* test). CLZ treatment did not significantly improve object recognition/discrimination in the *Map6*$_{+/-}$ mice (**B**) NAP treatment significantly improved object recognition/discrimination in the *Map6*$_{+/-}$ mice ($^{**}P < 0.01$, Student *t* test) to the control *Map6*$_{+/+}$ level (**C**) NAP+CLZ treatment significantly improved object recognition and discrimination in the *Map6*$_{+/-}$ mice ($^{**}P < 0.01$, Student *t* test) to the control *Map6*$_{+/+}$ mouse level (**D**). Although in (**B**), saline treatment appears to increase discrimination in comparison to the *Map6*$_{+/-}$ mice from (**A**), statistical analysis showed a *P* value of 0.073 (Student *t* test).

untreated mice (*Map6*$^{+/-}$ mice and *Map6*$^{+/+}$ mice; **Fig. 3A**) and 6 groups of treated *Map6*$^{+/-}$ mice as follows: s.c. injected 1) saline (Teva Medical, AWB 1324) and 2) CLZ (Sigma-Aldrich, C6305) in saline (**Fig. 3B**); intranasally administered 3) DD (7.5 mg of NaCl, 1.7 mg of citric acid monohydrate 3 mg of disodium phosphate dihydrate, and 0.2 mg of benzalkonium chloride solution [50%]/ml), the NAP vehicle[43,46]; 4) NAP (a kind gift from Allon Therapeutics) in DD (**Fig. 3C**) and combinations, each administered as above including 5) saline +DD and 6) NAP+CLZ (**Fig. 3D**).

Map6$^{+/-}$ mice were treated daily (5 d per wk) for 15 wk, and when a behavioral test was implemented, treatment was conducted 1 h prior to the experiment. The experiments were based on a large body of previous animal studies with NAP showing brain bioavailability after intranasal as well as systemic administrations and efficacy using treatment schedules of 5 d per wk.[43,59-61]

CLZ (3 mg/kg) was solubilized under acidic conditions (pH = ~2.0 with HCl, Merck, 1003171000)), and the solutions titrated back to ~pH 7.4 with NaOH (Merck, 1064981000). *Map6*$^{+/-}$ mice received daily doses of 3 mg/kg s.c. injection of CLZ (5 d a wk, 100 μl/d) for the duration of the experiment. Control *Map6*$^{+/-}$ mice were similarly injected with saline. It should be noted that most studies give 10 to 20 mg/kg/d CLZ, as we have done before.[43] However, here we wanted to test for a potential synergistic effect; therefore, we reduced the CLZ dose.

DD was used as a vehicle/placebo for NAP.[46,43] This placebo solution was administered to mice hand-held in a semi-supine position with nostrils facing the investigator. A pipette tip was used to administer 2.5 μl/nostril (total: 0.5 μg/5 μl/mouse/d). The mouse was hand held until the solution was fully absorbed (~10 s).

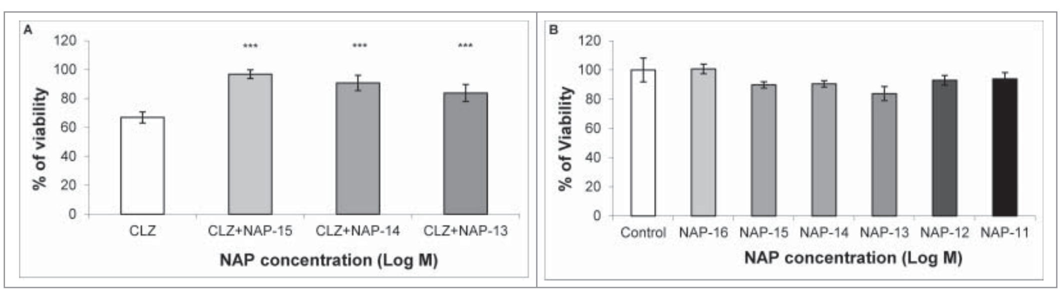

Figure 5. NAP protects against CLZ-associated cell death. In a neuronal cell culture model (human SH-SY5Y neuroblastoma) CLZ treatment resulted in approximately 40% decreased viability (**A**, CLZ vs. **B**, control, no CLZ). NAP treatment at 10^{-15}M to 10^{-13}M completely protected against CLZ-related reduction in viability. (NAP+CLZ vs. CLZ, one-way ANOVA, $F(3) = 103.618$, $P < 0.001$. The Fisher Least Significant Difference (LSD) post-hoc test revealed protection by NAP concentration of 10^{-15}M - 10^{-13}M: $^{***}P < 0.001$), (**A**). In the same neuronal cell model, without the addition of CLZ, at a wide concentration range (10^{-16}M to 10^{-11}M), NAP did not affect cell viability or cell division ($P > 0.05$), (**B**). However, the control group in (**B**) shows an 'unacceptably large variance', thus preventing the correct assessment of NAP effect. Experiments were repeated 3 times; one representative experiment is shown (n = 5 to 10/group/experiment).

Behavioral assessments were implemented according to our previous research.[43] In short, on the 4th wk of drug application, the open field test was conducted; on the 6th wk of drug application, an object recognition test was conducted. After 15 wk of treatment, the mice were decapitated, and hippocampal tissue was extracted and stored at −80°C for biochemical analysis. A second set of brains was used for immunohistochemistry as outlined below.

Biochemical Analyses

Extractions

RNA and proteins were extracted by the NucleoSpin® RNA/Protein kit (MACHEREY-NAGEL, REF 740933.50). The quantity and purity of RNA was determined by measuring optical density at 260 nm with a spectrophotometer (Nano-Drop Technologies, Wilmington, DE). RNA integrity was determined by electrophoresis on 1% agarose gel (Hispanagar, 45090001) and staining with ethidium bromide (Biolab, 05412399).

Reverse transcription and quantitative real-time PCR

Samples with the same amount of total RNA were used to synthesize single-strand cDNA using qScript™ cDNA Synthesis (Quanta BioSciences, Inc. 95047-100). Primer pairs were designed using the primer 3 web interface (http://frodo.wi.mit.edu/primer3/) and synthesized by Sigma-Genosys (The Woodlands, TX).

Adnp1: 5′ACGAAAAATCAGGACTATCGG3′; 5′GGACATTCCGGAAATGACTTT3′

Adnp2: 5′GGAAAGAAAGCGAGATACCG3′; 5′TCCTGGTCAGCCTCATCTTC3′

Becn1: 5′CAAATCTAAGGAGTTGCCGTT3′; 5′CTTCTTTGAACTGCTGCACAC3′

Hprt: 5′GGATTTGAATCACGTTTGTGTC3′; 5′AACTTGCGCTCATCTTAGGC3′

Real-time PCR reactions were carried out using PerfeCTa® SYBR® Green FastMix®, ROX™ (Quanta, 95073-012), and 300 nM each of forward and reverse primers. The StepOnePlus™ Real-Time PCR System Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA) was utilized according to the default thermocycler program for all genes. Data were analyzed with Data Assist™ v2.0 Software (Applied Biosystems).

The comparative Ct method was used for the quantification of transcripts, comparing the Ct value of the target gene to a housekeeping gene (*Hprt* [hypoxanthine phosphoribosyl transferase]) for each sample. The results were calculated as percent of 2^–ΔCT in the experimental sample compared to naïve *Map6*$^{+/-}$ mice, which constituted the 100% baseline.

Tissue preparation for histology and immunohistochemistry

At the end of the drug administration period, male mice (5 to 10/group) were perfused transcardially under deep anesthesia with 4% paraformaldehyde (Merck, k42072103) in 0.1 M phosphate-buffered saline, pH 7.4 (Biological Industries, 1407753). The brains were removed and placed in the same fixative at 4°C for 4 h and then immersed in 30% sucrose in phosphate-buffered saline, pH 7.4 for 5 d at 4°C.

Embedding

Brain tissues were embedded in tissue Tek and flash-frozen with liquid nitrogen. Subsequently, 6-μm serial sections were cut using a cryostat mtc (SLEE Mainz, Germany) microtome at −23°C, and sections were placed on superfrost plus slides and kept at −80°C until use.

Immunohistochemistry

Frozen sections were air dried for 1 h and then hydrated in distilled water. Endogenous peroxidase was blocked by incubating the sections in 3% H_2O_2 (Merck, k2769210) in methanol for 10 min. Sections were then rinsed with distilled water and then placed in tris-buffered-saline (containing 0.05 M Tris [Duchefa biochemie,T1501.1000], 0.15 M sodium chloride [Chemlab, CL00.1429.1000], and adjusted to pH 7.6 with 1 M HCl) for 5 min. After incubation in blocking buffer of 10% fetal bovine serum (Gibco, 10108-157) for 30 min, the sections were treated with primary antibodies against BECN1 (affinity-purified polyclonal rabbit IgG; Santa Cruz Biotechnology, sc11427). Secondary antibodies were goat anti-rabbit (Vector, BA-1000) and avidin-peroxidase (Sigma, A3151). Diaminobenzidine (Sigma, D12384) was used as chromogen. Nuclear counterstaining was performed with hematoxylin (Sigma, H9627).

Measurements

Sections were examined under an optical microscope (Zeiss Axioplan-2, Carl Zeiss Microscopy, LLC Thornwood, NY) with the aid of a CCD camera (Nikon DS-5M, Nikon Inc. Melville, NY). On average, 20 optical fields were examined from each experimental group under a magnification of 40×. Measurements were performed in the prefrontal cortex (Pr. Cortex) and in the hippocampus with ImageJ (1.43u). The results are expressed as cells/mm^2 and as Integrated Density (the product of Area and Mean Gray Value).

Human SH-SY5Y neuroblastoma cells as a model for CLZ toxicity and NAP protection

Cells were plated at a density of 3.6 × 10^5/well (96-well plates) 24 h prior to the CLZ treatment. Dulbecco's modified Eagle medium (Biological Industries, 01-170-1A) was supplemented with 15% fetal calf serum (Biological Industries, 04-102-2A), 1% penicillin-streptomycin (Biological Industries, 03-031-1), and 1% glutamine (Biological Industries, 03-020-1B) in a 5% CO_2 atmosphere. CLZ was obtained from Sigma (see above). Cells were exposed to NAP (10^{-15} M to 10^{-10} M) for 24 h at 37°C either in the absence or presence of 20 μg/ml CLZ. It should be noted here that this CLZ dose represents a 30- to 60-fold higher levels than the clinically optimal CLZ blood levels (300 to 600 ng/ml).[62]

Drugs and control solutions were dissolved in ethanol. The measurement of metabolic activity (which assesses cell survival) was performed by a nonradioactive cell proliferation assay (MTS; Promega, CellTiter96^R, G3580) and evaluated with an ELISA-reader (Bio-Tek Instruments, Highland Park, VT) at a wavelength of 490 nm.

Statistical analysis

One-way ANOVA with the Fisher least significant differences (LSD) post-hoc test on SigmaPlot software was used for multiple group comparisons. The 2-tailed Student *t* test was utilized when only 2 variables were compared, as outlined in the figure legends. Given the different vehicles tested, the comparisons were made between 2 different groups + vehicle (placebo) and + vehicle including treatment. Data were checked for outliers using the Grubbs test on GraphPad software. All determinations were made with a 95% confidence interval and were considered significant at the $P < 0.05$ level. All results are depicted as mean +/− standard error of the mean (SEM).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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