Haploinsufficiency of the autism candidate gene *Neurobeachin* induces autism-like behaviors and affects cellular and molecular processes of synaptic plasticity in mice

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ABSTRACT

Neurobeachin (NBEA), a brain-enriched multidomain scaffolding protein involved in neurotransmitter release and synaptic functioning, has been identified as a candidate gene for autism spectrum disorder (ASD) in four unrelated patients haploinsufficient for *NBEA*. The aim of this study was to map the behavioral phenotype of *Nbea*^{+/-} mice in order to understand its contribution to the pathogenesis of ASD. ASD-like behavioral variables of *Nbea*^{+/-} mice were related to basal neuronal activity in different brain regions by *in situ* hybridizations and extracellular field recordings of synaptic plasticity in hippocampal cornu ammonis 1 (CA1) region. Levels of BDNF and phosphorylated cAMP response element-binding protein (CREB) were measured in an attempt to investigate putatively underlying changes in these neuromolecules. *Nbea*^{+/-} mice exhibit several ASD-like features, including changes in self-grooming behavior, social behaviors, conditioned fear responses, and spatial learning and memory, which coincided with enhanced long-term potentiation (LTP) in their CA1 region. The observed alterations in learning and memory and hippocampal LTP are concomitant with decreased expression of the immediate early gene *zif268* in dorsomedial striatum and hippocampal CA1 region, increased CREB phosphorylation, and increased hippocampal BDNF expression. These findings indicate that *Nbea* haploinsufficiency leads to various molecular and cellular changes that affect neuroplasticity and behavioral functions in mice, and could thus underlie the ASD symptomatology in *NBEA* deficient humans.

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Introduction

With a prevalence of 0.6%, autism spectrum disorders (ASDs) are among the most common neurodevelopmental disorders. ASD is characterized by various pathognomonic behavioral alterations including impaired social interaction and communication as well as frequent intellectual disability and anxiety (Fombonne, 2009). It is

generally accepted that there is a strong genetic basis for ASD that probably involves multiple genes (Abrahams and Geschwind, 2008). Recently, *Neurobeachin* (*NBEA*) has been suggested as an ASD candidate gene following the description of a patient with *de novo* balanced reciprocal translocation *t*(5;13)(q12.1;q13.2) with a breakpoint in intron 1 of *NBEA* (Volders et al., 2011). In addition, three patients with a monoallelic *NBEA* deletion were reported. Initially, a woman with autism was described with a deletion involving 13q13 and portions of q12 and q14. This deletion was not present in an autistic maternal cousin (Ritvo et al., 1988). In the second patient, a paternally derived deletion encompassed a 9 Mb fragment in the 13q12–q13 region. The father was not further investigated for autistic features (Smith et al., 2002). A *de novo* deletion of 13q13.2–q14.1 was detected in a boy with autism in a screen for the incidence of cytogenetic abnormalities in a population of autism patients

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(Reddy, 2005). The *NBEA* gene is located in a 19 cM region that was identified as a candidate region for ASD by linkage study and encompasses a low-frequency common fragile site (*FRA13A*) linked to ASD (Volders et al., 2011).

Little is known about the function of *NBEA*, but it has been shown to be a brain-enriched multidomain scaffolding protein located at tubulovesicular endomembranes near the *trans*-Golgi network (TGN) in neuronal soma and dendrites. A small fraction of *NBEA* is present at the postsynaptic membrane of a minority of synapses (Wang et al., 2000). The C-terminal part of *NBEA* contains a pleckstrin homology – beige and Chediak-Higashi (BEACH) – WD40 domain module, generally implicated in vesicle trafficking (Gebauer et al., 2004; Jogl et al., 2002; Wang et al., 2000). The N-terminal region possesses a concanavalin A-like lectin domain flanked by Armadillo repeats suggested to play a role in intracellular trafficking (Breidenbach and Brunker, 2005; Burgess et al., 2009). Distal from these regions, an A kinase anchoring protein (AKAP) domain is present, recruiting cAMP-dependent protein kinase A (PKA) to endomembranes near the TGN by high affinity binding to its $R\text{II}\alpha$ subunit. *NBEA* has been shown to be involved in post-Golgi membrane trafficking and knockdown of *Nbea* in a neuroendocrine cell line led to enhanced secretion of dense-core secretory granules (DCSGs), the neuroendocrine counterpart of large dense-core vesicles (LDCVs) (Castermans et al., 2010). Furthermore, blood platelets of a patient haploinsufficient for *NBEA* showed abnormal morphology of dense granules (Castermans et al., 2010). Knockout *Nbea* in two independent mouse models resulted in perinatal lethality due to a complete block of evoked neuromuscular synaptic transmission (Medrihan et al., 2009; Su et al., 2004).

These findings suggest that *Nbea* haploinsufficiency may affect neural functioning in a subtle way, which could in fact explain the behavioral alterations observed in haploinsufficient patients. To test this hypothesis, we investigated whether *Nbea* haploinsufficiency in laboratory mice leads to changes in behavioral domains related to the diagnostic hallmarks of ASD.

We included tests of social behavior, since they relate to the core symptoms of ASD and only few ASD mouse models display such symptoms (Robertson and Feng, 2011), as well as tests for repetitive behavior, neuromotor performance, exploratory and general cage activity, sensitivity to auditory stimuli and sensorimotor gating, and learning and memory. Brain volume and regional neuronal activity were determined to examine whether *Nbea* haploinsufficiency affects gross brain morphology and function. As these analyses revealed changes in hippocampal activity, we assessed hippocampal (CA1) synaptic plasticity as well as some crucial plasticity-related molecular processes such as cAMP response element-binding protein (CREB) phosphorylation and levels of its target gene product brain-derived neurotrophic factor (BDNF), which might be at the basis of autistic-like and neurocognitive defects.

Materials and methods

Animals

The GH240B transgenic line (Su et al., 2004) was backcrossed for at least 10 generations with C57BL/6J mice (Janvier). Female mice of 20 weeks of age (17 *Nbea*^{+/+} and 18 *Nbea*^{+/-} mice) at the beginning of testing, were housed in groups in standard cages (wood-shaving bedding) under conventional laboratory conditions (12/12 h light/dark cycle, lights on at 8 am; 22 °C) and were allowed food and water *ad libitum*. Group-housed females were used to avoid territorial aggression and fighting that frequently occurs in males. Behavioral experiments were conducted during the light phase of their activity cycle. All procedures were approved by the ethical research committee of KU Leuven. The grooming test was performed with a new group of 20 week old female mice (12 *Nbea*^{+/+} and 10 *Nbea*^{+/-} mice). Body weight was monitored every 3 weeks. Body

and brain weight were further examined in 12 week old female mice ($n = 10/\text{group}$). Brains were isolated and immediately put on -80 °C before weighing.

Behavioral analysis

Ambulatory cage activity, accelerating rotarod, PPI, open field test, elevated plus maze, social exploration test, SPSN test, contextual fear conditioning and MWM test were each conducted according to the protocol earlier described (Goddyn et al., 2006; Nadler et al., 2004; Naert et al., 2011; Paradee et al., 1999; Piccart et al., 2011; Van Dam et al., 2000). The behavioral grooming test was modified from the protocol described in Berridge (1989). A detailed description of all behavioral tests is available in the supplementary information (SI).

In situ hybridizations for *zif268*

As previously established, ISH for *zif268* were performed on 25 μm coronal cryostat sections ($n = 4/\text{genotype}$) (van Brussel et al., 2009). A detailed ISH protocol can be found in SI. Briefly, air-dried sections and a set of standard [¹⁴C] microscopes (GE Healthcare, UK) were exposed to an autoradiographic film (Kodak, Belgium). Densitometric analysis of the digitized autoradiographic images was performed using the ImageJ imaging system (National Institutes of Health, Bethesda, Maryland, USA). The optical density (OD) of each region of interest was expressed as mean gray value per pixel. OD measurements of prelimbic cortex (PL), anterior cingulate cortex (aCC), striatum, hippocampus and amygdala were determined in consecutive brain slices along the rostrocaudal axis, respectively from +1.78 mm till +1.54 mm, +1.42 mm till +1.10 mm, +1.54 mm till +1.10 mm, –1.22 mm to –2.30 mm and –1.46 mm to –2.06 mm relative to bregma (Franklin, 2008). In the striatum, OD was determined for 3 sub-regions, dorsomedial (DMS), dorsolateral (DLS) and ventral striatum (VS), while in the hippocampus a detailed quantification of CA1 and CA3 was also performed.

Electrophysiological recordings

For electrophysiological experiments, 27- to 30-week-old female mice (same age as mice during MWM experiment) ($n = 6/\text{group}$) were killed by cervical dislocation. Details concerning the preparation of the transverse hippocampal slices and the recording conditions can be found in SI.

RNA interference of *Nbea* in βTC3 cells

The mouse pancreatic βTC3 cell line was grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2 . Lipofectamine RNAiMAX (Life Technologies, UK) was used to transiently transfect βTC3 cells with either 40 nM siGENOME SMARTpool siRNA mouse *Nbea* or stealth RNAi siRNA negative control medium GC (Thermo Fisher Scientific, Illinois, USA). Cells were harvested after 72 h and divided for immunoblotting or determination of *Nbea* knockdown. The knockdown of *Nbea* was assessed by means of a quantitative PCR. RNA of transfected cells was extracted with Nucleospin RNA II (Macherey-Nagel, Germany) and cDNA synthesis was performed with iScript cDNA synthesis kit (Bio-Rad, California, USA). The mRNA expression of *Nbea* was calculated using the method of Livak with normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene (Livak and Schmittgen, 2001). The following primers were used: *Nbea*_F 5' CTTTGTGCGGATCAACAGG 3'; *Nbea*_R 5' CATTGATGGAGTTGGCAAGA 3'; GAPDH_F 5' ATGGCCTTCCG TGTTCCT 3'; GAPDH_R 5' CAGCGGCACGTCAGAT 3'.

Immunoblotting

Standard Western blotting techniques were used to quantify CREB and pCREB relative to actin. For details concerning Western blot procedure see SI. Quantification was performed using Kodak Imager Software (Kodak, New York, USA) and the expression level in cells transfected with control siRNA was set at 100%.

Quantification of BDNF levels in hippocampus

Hippocampi were dissected from 8 week old mice ($n = 7/\text{genotype}$) and immediately put on -80°C . Tissue was homogenized in RIPA buffer (25 mM Tris-HCl pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS) containing a protease and phosphatase inhibitor cocktail (Roche, Belgium). BDNF levels were assessed by means of the Mouse BDNF ELISA kit (Abnova, Taiwan) according to manufacturer's instructions. Protein concentration was determined with BCA protein assay (Thermo Fisher Scientific, Illinois, USA) allowing normalization of BDNF content to total protein content.

Statistical analysis

Data are presented as mean with standard error of the mean (SEM). Significance of differences between mean values were analyzed using (where appropriate) two-tailed t test, Mann-Whitney U test (MWU), analysis of variance (ANOVA) or repeated measures (RM) ANOVA procedures with Tukey test *post hoc* comparison using Statistica version 9.0 (StatSoft Inc., Oklahoma, USA). All statistical tests were performed with 0.05 as the α level of significance (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). The data from one excessively overweight Nbea^{+/-} mouse were excluded from the analyses.

Results

Increased relative brain volume in Nbea^{+/-} mice

The body weight of Nbea^{+/-} mice was significantly lower than Nbea^{+/+} mice (RM-ANOVA: main effect of genotype: $F(1,32) = 39.8$, $P < 0.001$) and this difference remained constant during behavioral testing (Fig. S1A). Further investigation in 12 week old female mice revealed reduced body and brain weight in Nbea^{+/-} mice compared to Nbea^{+/+} mice ($t = 9.80$; $P < 0.001$ and $t = 5.27$; $P < 0.001$ respectively) resulting in an elevated brain to body weight ratio ($t = -8.60$; $P < 0.001$) (Figs. S1B–D). Since Nbea^{+/-} mice are small in stature, this was taken into account in all behavioral analyses.

Diminished ambulatory activity in Nbea^{+/-} mice during night-day transition

Cage activity recording during a 23 h period showed an increase in activity for both groups shortly after introduction into the cage, but during the subsequent hours of testing, there was no significant difference between wildtype and heterozygous mice (RM-ANOVA: main effect of genotype: $F(1,32) = 1.58$, $P = 0.22$). However, RM-ANOVA showed a particular decrease in horizontal ambulatory cage activity in Nbea^{+/-} mice compared to Nbea^{+/+} mice during the sunrise period (RM-ANOVA: main effect of genotype: $F(1,32) = 8.36$, $P = 0.007$) (Fig. S2). The accelerating rotarod revealed no significant differences between Nbea^{+/-} mice and Nbea^{+/+} mice (RM-ANOVA: main effect of genotype: $F(1,32) = 0.02$, $P = 0.89$). A learning curve was observed over the testing trials in both groups (RM-ANOVA: main effect of trial: $F(3,81) = 9.44$, $P < 0.001$) (Fig. S3).

Nbea^{+/-} and Nbea^{+/+} mice display similar exploratory activity

In the open field test, there was no significant difference between the total path length of Nbea^{+/-} and Nbea^{+/+} mice ($t = -1.72$, $P = 0.096$) (Fig. 1B). Both groups travelled more in the periphery compared to the center circle (main effect of zone: $F(1,64) = 228.38$; $P < 0.001$) and there were no significant differences between Nbea^{+/-} and Nbea^{+/+} mice in this respect. Anxiety-related exploration in the elevated plus maze also failed to reveal differences between Nbea^{+/-} and Nbea^{+/+} mice as both groups showed a preference for visiting the closed arms (main effect of arm: $F(1,32) = 152.1$, $P < 0.001$) and similar total beam crossings ($t = 1.09$, $P = 0.29$) (Fig. S4).

Nbea^{+/-} mice show normal sensorimotor gating

All mice exhibit normal auditory capacity except for one wildtype mouse that was excluded for further analysis. Habituation effects were observed across the three trial blocks in startle amplitude (RM-ANOVA: main effect of trial block: $F(2,62) = 10.77$, $P < 0.001$) but there was no effect of genotype ($F(1,31) = 1.44$, $P = 0.24$) or trial block $\times$ genotype interaction ($F(2,62) = 0.23$, $P = 0.80$) (Fig. S5A). Prepulse inhibition (PPI) revealed similar reactions of wildtype and heterozygous mice to all prepulses (RM-ANOVA: main effect of genotype: $F(1,31) = 1.37$, $P = 0.25$) with a main effect of prepulse intensity ($F(4,124) = 3.28$, $P = 0.014$) (Fig. S5B).

Increased self-grooming behavior of Nbea^{+/-} mice

As a measure for repetitive behavior, self-grooming was assessed after an aversive stimulus on the head of the test mouse. Heterozygous Nbea mice showed increased self-grooming behavior after their snout was sprayed with water mist compared to wildtype mice ($t = -3.49$; $P = 0.002$) (Fig. 1A).

Altered social behaviors in Nbea^{+/-} mice

In the social exploration test, the forced presence of two stranger mice in the center of the arena resulted in an enhanced overall ambulatory activity of Nbea^{+/-} mice (main effect of genotype: $F(1,56) = 16.1$, $P < 0.001$) leading to an increased path length in the center of the arena (*post hoc* comparison: $P = 0.008$; Fig. 1C). Other approach measures were not different between the groups.

This indication of altered social behavior was further confirmed in the sociability and preference for social novelty (SPSN) test. In the first SPSN session, the social preference trial, the test mouse was given the choice to enter a side chamber containing an unfamiliar mouse (Stranger1) or a side chamber containing an empty cage. RM-ANOVA revealed a main effect of chamber ($F(1,32) = 6.07$, $P = 0.019$) with wildtype mice exhibiting a preference for the chamber containing the unfamiliar mouse (Stranger1) over the empty cage (*post hoc* comparison: $P = 0.014$) whereas Nbea^{+/-} mice spent equal time in both chambers (Fig. 2A). Notably, during both sessions, the total distance travelled did not significantly differ between Nbea^{+/+} and Nbea^{+/-} mice (session 1: $U = 124.5$; $P = 0.51$ and session 2: $U = 126$; $P = 0.55$). The actual social interaction of Nbea^{+/-} mice with the stranger mouse, expressed as sniff time, only occurred during the first time period after exposure to the new chambers (*post hoc* comparison; $P < 0.001$), whereas Nbea^{+/+} mice interacted more with the stranger mouse during the first three time periods with significant *post hoc* comparison for time period 1 ($P = 0.009$) and time period 2 ($P = 0.03$) (Figs. 2B–C). During the second session of the test (the preference for social novelty trial), both Nbea^{+/-} and Nbea^{+/+} mice showed preference for the chamber containing the unfamiliar mouse (Stranger2) over the familiar mouse (Stranger1) (main effect of chamber: $F(1,32) = 10.05$, $P = 0.003$ with *post hoc* comparison for Nbea^{+/+} mice: $P = 0.048$ and Nbea^{+/-} mice: $P = 0.021$) (Fig. 2D). Both wildtype and heterozygous

Fig. 1. Self-grooming, open field and social exploration test of Nbea^{+/-} mice and their wildtype littermates. (A) Compared to wildtype mice, Nbea^{+/-} mice displayed a significant increase in self-grooming behavior after spraying with water mist on their face. (Nbea^{+/+} mice: $n = 12$, Nbea^{+/-} mice: $n = 10$) (B) In the open field test, Nbea^{+/-} mice had a similar exploratory pattern as Nbea^{+/+} mice as both travelled comparable path length in the center and periphery. (C) The presence of wired stranger mice in the social exploration test induced an increased overall exploration of the Nbea^{+/-} mice leading to an increased exploration in the center and periphery. (Nbea^{+/+} mice: $n = 16$, Nbea^{+/-} mice: $n = 18$).

mice had increased sniff time with stranger 2 in the first 2 time periods with a significant *post hoc* comparison for Nbea^{+/+} mice in the first time period ($P = 0.022$) (Figs. 2E–F).

Haploinsufficiency of Nbea enhances conditioned fear responses and impairs spatial learning and memory performance

In the contextual fear conditioning test, heterozygous mice showed overall increased percentage of freezing (RM-ANOVA: main effect of genotype: $F(1,32) = 6276.7$, $P < 0.001$). When exposed to the same context after aversive footshock conditioning, Nbea^{+/-} mice displayed an increased percentage of freezing compared to Nbea^{+/+} mice (*post hoc* comparison: $P < 0.001$) (Fig. 3). This difference could not be caused by increased baseline freezing behavior since there was no significant effect of genotype after initial exposure to the shock (*post hoc* comparison: $P = 0.98$). Exposure to a new context (pre-CS) induced hardly any freezing, but presentation of the auditory stimulus resulted in a marked increase in percentage of

freezing for both genotypes with no significant difference between wildtype and heterozygous mice (*post hoc* comparison: $P = 0.12$) (Fig. 3).

During the acquisition trials of the Morris water maze (MWM) test, Nbea^{+/-} mice travelled an overall increased distance which is represented as path length in Fig. 4A (RM-ANOVA: main effect of genotype: $F(1,32) = 9.20$; $P = 0.005$). In the first probe trial, heterozygous mice failed to show a preference for the target quadrant compared to wildtype mice (interaction quadrant $\times$ genotype: $F(3,128) = 2.78$, $P = 0.044$). This lack of spatial preference in the Nbea^{+/-} group during the first probe trial was also illustrated by heat plots where the Nbea^{+/-} mice demonstrates to search in 4 quadrants of the pool while Nbea^{+/+} mice already showed preference for the platform quadrant (Fig. 4B). Notably, the reduced bodyweight of the Nbea^{+/-} mice did not change their swimming velocity. In the second probe trial, performed after one more week of training, Nbea^{+/-} mice did develop a preference for the target quadrant but less pronounced than Nbea^{+/+} mice (interaction quadrant $\times$ genotype: $F(3,128) = 4.74$; $P = 0.0036$ with *post hoc* comparison: $P = 0.020$) (Fig. 4B).

Fig. 2. Time spent in a chamber in the SPST test with time period analysis of social interaction. (A) In the sociability trial, wildtype mice showed a clear preference in spending time with a stranger mouse whereas heterozygous mice spent equal time in both side chambers. (B–C) This reflects the social interaction, measured as time spent sniffing, for both genotypes. Nbea^{+/+} mice showed a significantly increased social interaction during the first 6 minutes of the sociability trial compared to the first 2 minutes of the Nbea^{+/-} mice. (D) During the preference for social novelty trial, both Nbea^{+/+} mice and Nbea^{+/-} mice had a preference for the unfamiliar mouse (Stranger2) as they both spent more time in the chamber containing Stranger2. (E–F) Equal results were seen for the social interaction. Wildtype and heterozygous mice showed significantly increased social sniffing during the first 2 time periods. (Nbea^{+/+} mice: $n = 16$, Nbea^{+/-} mice: $n = 18$).

Fig. 3. Contextual fear conditioning test of wildtype and heterozygous mice. Nbea^{+/-} mice showed significant increased percentage of freezing after exposure to the same context. This cannot be explained by an increased baseline freezing behavior since no significant difference of freezing in the shock condition was present. In the conditioning phase, Nbea^{+/-} mice displayed a tendency for higher freezing behavior after hearing the conditioned stimulus however the *post hoc* comparison was not significant. This tendency was not due to a shift in baseline freezing because no significant difference in freezing behavior was observed in the pre-conditioned stimulus phase. (Nbea^{+/+} mice: $n = 16$, Nbea^{+/-} mice: $n = 18$).

Decreased expression of *zif268* mRNA in the dorsomedial striatum and hippocampal CA1 of Nbea^{+/-} mice

The expression level of *zif268* mRNA was decreased in DMS of Nbea^{+/-} mice ($t = 2.35$, $P = 0.020$) (Figs. 5A, C), but no significant difference was detected for DLS and VS (Fig. S6A). In addition, reduced expression levels of *zif268* mRNA in the total hippocampal formation ($t = 2.91$, $P = 0.005$) as well as a marked decrease in expression in the CA1 region ($t = 2.17$, $P = 0.033$) but not in CA3 region was found in Nbea^{+/-} mice (Figs. 5B–C and S6B). Expression of *zif268* in medial prefrontal cortex (PL and aCC) and amygdala did not differ between Nbea^{+/-} and Nbea^{+/+} mice (Fig. S6C–D).

Enhanced long-term potentiation in CA1 area of the hippocampus in Nbea^{+/-} mice

Extracellular recordings in the CA1 area of hippocampus indicated normal basic synaptic excitability in Nbea^{+/-} mice since the I/O curves did not differ between Nbea^{+/+} mice and Nbea^{+/-} mice (Fig. 6A). Likewise, Nbea^{+/-} mice did not show alterations in presynaptically mediated short-term plasticity as proven by similar ratios of PPF as compared to their wildtype littermates (Fig. 6B). LTP, however, was evidently improved. Slices of Nbea^{+/-} mice attained a significantly higher initial magnitude of potentiation as compared to Nbea^{+/+} mice (1 min: Nbea^{+/-} mice: $264.0 \pm 27.8\%$; Nbea^{+/+} mice: $185.0 \pm 25.2\%$, MWU: $P = 0.034$). Since the decay of potentiation was similar in both genotypes this resulted in a main effect of genotype (RM-ANOVA: $F(1,14) = 4.62$; $P = 0.04$) (Fig. 6C).

Nbea deficiency leads to increased phosphorylation of CREB in vitro and higher BDNF levels in vivo

Another mouse model that displayed exorbitant LTP combined with a suboptimal performance in the MWM task is the transgenic VP16-CREB mouse, a model for constitutively active CREB (Viosca et al., 2009). CREB is activated upon phosphorylation of serine 133, a demonstrated target-site of PKA (Gonzalez and Montminy, 1989). Since Nbea is an AKAP protein and defective sequestering of inactive PKA has been shown to result in increased PKA activity (Corstens et al., 2006) this might lead to changes in the pCREB/CREB ratio. Therefore, the phosphorylation status of CREB was examined in the neuroendocrine cell line β TC3, after knockdown of Nbea. The expression of Nbea was reduced with $42\% \pm 5.8\%$ which resembles the

Fig. 4. Learning and memory assessment in the Morris water maze test. (A) During the acquisition trials, Nbea^{+/-} mice travelled increased path length in order to find the hidden platform as detected by RM-ANOVA but *post hoc* comparison revealed no significant difference. (B) In the first probe trial, wildtype mice were able to successfully find the location of the platform, measured as distance covered in the target quadrant, whereas Nbea^{+/-} mice did not show a preference for the target quadrant. In the second probe trial both Nbea^{+/+} mice and Nbea^{+/-} mice swam more in the target quadrant but wildtype mice were more efficient in locating the hidden platform. Heat plots per genotype per probe trial indicate the time each group spent on a specific position with a warmer color for longer time periods. These heat plots confirmed that Nbea^{+/-} mice were not able to locate the platform in the first probe trial. In probe trial 2, Nbea^{+/-} mice had developed a preference in spending time in the target quadrant but to lesser extent than Nbea^{+/+} mice. The heat plots illustrated that the performance of Nbea^{+/-} mice in the second probe trial was of the same level as Nbea^{+/+} mice in the first probe trial. (Nbea^{+/+} mice: $n = 16$, Nbea^{+/-} mice: $n = 18$).

haploinsufficient status of Nbea^{+/-} mice. Quantitative analysis of 3 independent experiments indicated that Nbea haploinsufficiency did not alter the total amount of CREB ($t = 0.87$; $P = 0.43$) but significantly increased the phosphorylation of CREB (factor 2.35) ($t = -4.02$; $P = 0.016$) (Fig. 7A). Several attempts to extrapolate these data to the hippocampus failed because pCREB was too low for direct detection and quantification. To overcome this issue, the levels of BDNF were determined in 8 week old hippocampi as BDNF is a target of pCREB (Barco et al., 2005) and reaches the highest expression level at the age of 8 weeks. The hippocampus of Nbea^{+/-} mice contained 32% more BDNF compared to Nbea^{+/+} mice ($t = -2.28$; $P = 0.041$) which is indicative for a higher pCREB/CREB ratio in the hippocampus (Fig. 7B).

Discussion

Nbea^{+/-} mice displayed several phenotypic changes directly or indirectly related to ASD symptoms. Haploinsufficiency of Nbea increased self-grooming behavior, decreased sociability read-outs, enhanced conditioned fear, and impaired spatial learning and memory. These findings coincided with increased relative brain volume and

Fig. 5. *In situ* hybridizations for *zif268* in striatum and hippocampus. (A) Brain sections of Nbea^{+/+} mice and Nbea^{+/-} mice with the DMS indicated with a dotted line. (B) Brain sections of Nbea^{+/+} mice and Nbea^{+/-} mice with the CA1 region of the hippocampus indicated with a dotted line. (C) Quantification of *zif268* expression by ImageJ imaging system. Both DMS and CA1 region of Nbea^{+/-} mice had a decreased expression of *zif268* indicating a decreased neuronal activity in those regions. (Nbea^{+/+} mice: *n* = 4, Nbea^{+/-} mice: *n* = 4).

alterations in DMS and hippocampus activation (based on *zif268* ISH) and enhanced hippocampal LTP. At the molecular level, Nbea knock-down in β TC3 cells resulted in increased phosphorylation of CREB, and higher levels of its target gene product BDNF in the hippocampus of Nbea^{+/-} mice.

Prepulse inhibition is commonly used to test sensorimotor gating, which is often impaired in patients with ASD or schizophrenia (Perry et al., 2007). We failed to observe any defect but C57BL/6J mice have one of the lowest PPI levels, which complicates the detection of impairments (Crawley et al., 1997).

Several other behavioral tests showed changes in Nbea^{+/-} mice that might relate to characteristic behavioral symptoms of ASD, especially those of patients with NBEA deficiency (Castermans et al., 2003). Nbea^{+/-} mice showed an increase in self-grooming behavior after their snout was sprayed with water mist. Tests of repetitive behavior are often executed in the context of obsessive-compulsive disorder or ASD (Wang et al., 2011; Welch et al., 2007). Increased self-grooming behavior of Nbea^{+/-} mice might be indicative for impairments in repetitive behavior (Silverman et al., 2010). Moreover, Nbea^{+/-} mice displayed changes during social exploration and the SPSN test. Sociability read-outs of the SPSN task might be the most relevant autism-like symptoms in mice (Yang et al., 2011). Therefore, diminished sociability in Nbea^{+/-} mice may relate to social approach deficits of autistic patients (Silverman et al., 2010). Reduced sociability could not be attributed to changed exploration, neuromotor performance, or anxiety or innate fear, since Nbea^{+/-} mice performed normally in open field, rotarod and elevated plus maze tests (Moy et al., 2009). Although Nbea^{+/-} mice show defects in social approach and social exploration, their preference for social novelty trial was normal. Thus, Nbea^{+/-} mice are able to distinguish a novel mouse from a previously encountered mouse, which is reminiscent of behavioral changes in other ASD mouse models.

Fig. 6. Electrophysiological recordings in CA1 of the hippocampus. (A) Based on I/O curves, basic synaptic synaptic excitability was normal in Nbea^{+/-} mice at all stimulus intensities measured (Nbea^{+/-} mice: *n* = 7; Nbea^{+/+} mice *n* = 9). (B) PPF did not differ between Nbea^{+/+} and Nbea^{+/-} mice, regardless of the inter pulse interval, indicating normal short-term presynaptic plasticity (Nbea^{+/-} mice: *n* = 8; Nbea^{+/+} mice *n* = 9). (C) LTP was significantly increased in Nbea^{+/-} mice after 3 × TBS detected by RM-ANOVA. (Nbea^{+/+} mice *n* = 9, Nbea^{+/-} mice: *n* = 7).

Increased brain/body weight ratio is a common feature of ASD present in approximately 20% of ASD individuals (McCaffery and Deutsch, 2005; Stigler et al., 2011). Although Nbea^{+/-} mice are smaller in stature due to selective inhibition of postnatal pituitary development (Su et al., 2004), this cannot account for the increased relative brain volume as growth hormone deficiency generally leads to a decreased brain volume (Segal et al., 2004). Of notice, Olszewski et al. (2012) observed no dwarfism in Nbea^{+/-} mice generated by means of the gene-trap vector system (Olszewski et al., 2012). This discrepancy might be due to the different methods used to generate the mouse models. A possible hypothesis of the basis of the detected brain overgrowth is the elevated BDNF level in the hippocampus as mature BDNF regulates neuronal structure whereas the precursor proBDNF mediates apoptosis (Lee et al., 2001). The elevated level of BDNF in the hippocampus might be indicative for an overall increased level of BDNF. *In vitro*, Nbea has been identified as a negative regulator of the regulated secretion (Castermans et al., 2010). Defects in fine-tuning of vesicular secretion could lead to increased blood levels of BDNF, which has been reported in several patients and mouse models for ASD (Nickl-Jockschat and Michel, 2011). Moreover, NBEA has recently been identified in a screen for proteins important for neuronal differentiation (Iwaniuk et al., 2010).

No differences in *zif268* expression were detected in aCC and amygdala, two regions known to be important for social processing, but we did identify reduced expression in hippocampus and DMS. Defects in *zif268*-controlled signaling in hippocampus and striatum are likely to affect hippocampus-prefrontal (the extensive connections between the ventral part of aCC and hippocampus) and corticostriatal pathways that are crucial for social behavior as well as various neurocognitive functions. Recently, it has been shown that Nbea is required for dendritic spine formation, likely by regulating the assembly of F-actin (Niesmann et al., 2011). These findings were established in

Fig. 7. Measurement of phosphorylation of CREB *in vitro* and BDNF levels *in vivo*. (A) Total amount of CREB in β TC3 cells was unaltered after knockdown of Nbea whereas the proportion of pCREB increased with factor 2.35. Levels of CREB and pCREB were normalized first against actin and the expression level in cells transfected with control siRNA was set at 100% ($n=3$). (B) The BDNF level was first normalized to the total protein content of the sample and BDNF level of wildtype hippocampi was set at 100%. The BDNF levels in heterozygous hippocampi were 32% higher. (Nbea^{+/+} mice $n=7$, Nbea^{+/-} mice: $n=7$).

cultures of cortical neurons from Nbea^{+/-} mice, providing further evidence for defective corticostriatal signaling. In addition, Nbea has been suggested to be involved in plasticity of the visual cortex (Rietman et al., 2012).

Nbea^{+/-} mice clearly showed impaired learning and memory in MWM test. They displayed delayed spatial learning, as they were unable to find the hidden platform during the first probe trial, but succeeded in locating it during the second probe trial. Apparently, Nbea^{+/-} mice were able to overcome these deficits in spatial learning ability at least partly with prolonged training. Impaired but partially preserved learning and memory abilities were also found in other ASD models (Robertson and Feng, 2011), and spatial working memory is impaired in adolescents and adults with ASD (Williams et al., 2005).

Changes in hippocampal LTP could explain the poor performance of Nbea^{+/-} mice in the MWM test. Accumulating evidence suggests that enhanced LTP can have detrimental consequences on learning and memory performance (D'Hooge et al., 2005; Jolas et al., 2002; Nguyen et al., 2000; Parent et al., 1999) and hippocampal place-cell functions (Taverna et al., 2005). VP16-CREB mice with constitutively active CREB, also displayed normal I/O curves and PPF with enhanced LTP and suboptimal learning in MWM task (Barco et al., 2002; Viosca et al., 2009). Facilitation of LTP might result in an unfavorable shift of synaptic weights in neuronal networks during learning, saturating these networks and causing reduced learning capacity (Lopez de Armentia et al., 2007; Viosca et al., 2009). We found that Nbea^{+/-} mice also showed enhanced context-dependent fear responses. The change in learned fear could again be part of the ASD-like phenotype of Nbea^{+/-} mice. Two mouse lines with moderate levels of constitutively active CREB showed enhanced context freezing in a comparable protocol for aversive footshock conditioning (Suzuki et al., 2011). This suggests that increased pCREB levels might be at the basis of the learning and memory defects in Nbea^{+/-} mice.

Nbea^{+/-} mice displayed reduced levels of *zif268* in hippocampal CA1 and DMS, which are two brain regions crucial for spatial learning and memory (D'Hooge and De Deyn, 2001; Dillon et al., 2008). This change in levels of *zif268*, an immediate early gene in spatial memory processes (Guzowski et al., 2001), could contribute to the learning and memory defects observed here. While pCREB plays an important role in induction and maintenance of LTP, *zif268* plays a critical role in the stabilization of late LTP (Bozon et al., 2003; Jones et al., 2001), and *zif268* knockout mice were unable to maintain stable place fields in CA1 region (Renaudineau et al., 2009). Both pCREB and the downstream expression of *zif268* are required in hippocampus and striatum for

spatial memory formation (Porte et al., 2008). Furthermore, disturbed hippocampal or striatal signaling can be consistent with changes in learned fear since enhanced or exaggerated fear responses have been observed in juvenile mice, and attributed to immaturity of hippocampus-prefrontal pathways (Ito et al., 2009). CREB activation probably has a key role in delineating these learning-dependent circuits, and the decreased *zif268* levels in DMS and hippocampus of Nbea^{+/-} mice may have resulted from increased CREB phosphorylation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nbd.2012.11.004>.

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