

Cholinergic cells in the nucleus basalis of mice express the *N*-methyl-D-aspartate-receptor subunit NR2C and its replacement by the NR2B subunit enhances frontal and amygdaloid acetylcholine levels

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It is known that glutamatergic and cholinergic systems interact functionally at the level of the cholinergic basal forebrain. The *N*-methyl-D-aspartate receptor (NMDA-R) is a multiprotein complex composed of NR1, NR2 and/or NR3 subunits. The subunit composition of NMDA-R of cholinergic cells in the nucleus basalis has not yet been investigated. Here, by means of choline acetyl transferase and NR2B or NR2C double staining, we demonstrate that mice express both the NR2C and NR2B subunits in nucleus basalis cholinergic cells. We generated NR2C-2B mutant mice in which an insertion of NR2B cDNA into the gene locus of the *NR2C* gene replaced NR2C by NR2B expression throughout the brain. This NR2C-2B mutant was used to examine whether a subunit exchange in cholinergic neurons would affect acetylcholine (ACh) content in several brain structures. We found increased ACh levels in the frontal cortex and amygdala in the brains of NR2C-2B mutant mice. Brain ACh has been implicated in neuroplasticity, novelty-induced arousal and encoding of novel stimuli. We therefore assessed behavioral habituation to novel environments and objects as well as object recognition in NR2C-2B subunit exchange mice. The behavioral analysis did not indicate any gross behavioral alteration in the mutant mice compared with the wildtype mice. Our results show that the NR2C by NR2B subunit exchange in mice affects ACh content in two target areas of the nucleus basalis.

Keywords: Acetylcholine, behavioral habituation, NMDA receptors, novelty, NR2C-2B mice, nucleus basalis

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N-methyl-D-aspartate receptors (NMDA-Rs) have been implicated in neuroplasticity and memory processes (Morris *et al.* 2003). Native NMDA-Rs are composed of an NR1 and at least of one of four NR2 (A–D) and/or NR3 subunits. These subunits are encoded by separate genes, which show distinct developmental, brain regional, cellular and subcellular expression patterns (Das *et al.* 1998; Hollmann & Heinemann 1994). The functional characteristics of the NMDA-R are determined by their subunit composition (Dingledine *et al.* 1999). We generated mice in which the coding sequence of the *NR2C* gene was replaced by the one of the *NR2B* gene, whereas the regulatory sequences of the *NR2C* gene were kept functional (Schlett *et al.* 2004). These mice express the NR2B subunit instead of the NR2C subunit throughout the brain. Consequently, the NR2C protein was absent in cerebellar slices of adult mutant mice, whereas their level of the NR2B protein was increased. Compared with NMDA-Rs bearing the NR2C subunit, those featuring the NR2B subunit have several distinct properties, e.g. a higher Ca²⁺ conductance, higher Mg²⁺ sensitivity, higher excitatory postsynaptic potentials (EPSPs) and coupling to distinct second messenger systems (Nakanishi & Masu 1994). Consequently, in cerebellar slices, the amplitude of NMDA currents was increased in NR2C-2B subunit exchange mice. We also found aberrations in cerebellar morphology together with motor deficits on an accelerating rotarod in these mice (Schlett *et al.* 2004).

The cholinergic basal forebrain innervates various limbic and cortical areas and plays an important role in cognitive functions (Sarter & Bruno 2000). The infusion of NMDA into the basal forebrain induces cortical acetylcholine (ACh) release (Fournier *et al.* 2004), and NMDA-R in the basal forebrain contributes to the release of ACh after behavioral stimulation (Fadel *et al.* 2001). The NMDA-R subunit composition of cholinergic cells in the nucleus basalis has not been investigated so far, neither in rats nor in mice. Therefore, we examined whether the NR2B or NR2C subunits are expressed in these cells.

NMDA-R antagonists given systemically have been shown to impair behavioral habituation to an open field (Dai & Carey 1994) and object memory (Baker & Kim 2002; Packard & Teather 1997). Deficits in object memory have been reported after the infusions of NMDA-R antagonists into the perirhinal

cortex (Abe & Iwasaki 2001) and hippocampus (Baker & Kim 2002). The overexpression of the NR2B subunit in the fore-brain improved object memory after long retention intervals up to 3 days (Tang *et al.* 1999). We therefore examined whether spatial and object recognition is likewise improved in NR2C-2B mice.

Brain ACh has been implicated in behavioral habituation to novel environments (Schildein *et al.* 2000) and object recognition (Abe & Iwasaki 2001). The exposure of rats to spatial novelty increased the extracellular levels of hippocampal ACh. This increase was positively correlated with the amount of exploratory behavior exerted (Thiel *et al.* 1998). ACh receptor (ACh-R) activation enhances NMDA-mediated responses (Sabatino *et al.* 1999). Interactions between cholinergic and NMDA receptors also occur during neural plasticity (Broide & Leslie 1999; Gothert & Fink 1989). Parallel to NMDA antagonism, interventions into cholinergic systems influence spatial learning (Whishaw & Tomie 1987). Therefore, we determined whether the NR2C-2B subunit exchange has affected ACh concentrations in different brain regions of NR2C-2B mice in order to relate changes in ACh content to possible changes in behavioral habituation to novel environments and object recognition. Because the exploration of novel environments and objects correlates with emotionality (Pawlak & Schwarting 2002) and because NMDA-R subunits have been implicated in experimental anxiety (Dere *et al.* 2003a), we investigated whether the NR2C-2B subunit exchange has an effect on elevated plus-maze behavior.

Materials and methods

Animals

The generation of the NR2C-2B subunit exchange mice and the verification of successful subunit exchange by *in situ* hybridization and Western blotting methods as well as electrophysiological and anatomical examinations of the NR2C-2B mice are described elsewhere (Schlett *et al.* 2004). Male NR2C-2B substitution mice ($n = 10$) and wildtype littermates ($n = 9$) (2 months old) (obtained from the animal breeding division of the Institute for Cellular Biology and Immunology, University of Stuttgart, Germany) with a C57BL/6 genetic background were used for behavioral and biochemical tests. One week prior to behavioral experimentation, the mice were single-housed in standard Makrolon cages and had continuous access to food (10H10; Nohrlin, Bad Salzflun, Germany) and tap water. The acclimatization period to the housing conditions during the experiment was 1 week, and during this time, the animals were handled daily. They were maintained on a 12-h light/dark cycle, with lights switched on at 0700 h and were tested during the light phase between 0900 h and 1600 h. The order of behavioral testing was (a) open field, (b) object exploration and (c) elevated plus maze. All experiments were performed according to the guidelines of the German Animal Protection Law

and were approved by the North Rhine-Westphalia state authority.

Immunocytochemistry

We examined whether the wildtype mice express the NR2C or NR2B protein in cholinergic neurons of the nucleus basalis, by means of double *in situ* hybridization using fluorescence-labeled probes against choline acetyl transferase (ChAT) and NR2B, or NR2C, respectively. Furthermore, a possible coexpression of the NR2B protein along with ChAT in nucleus basalis neurons was determined for NR2C-2B subunit exchange mice.

Cloning mouse ChAT

Mouse ChAT cDNA was cloned from tissue RNA by RT-PCR using random hexamer primers. The PCR steps included 5 min at 95 °C, followed by 35 cycles: 30 seconds, 95 °C; 30 seconds, 60 °C; 60 seconds, 72 °C and a final elongation step for 10 min at 72 °C. The primer sequences for ChAT are 5'-GCCTGGTATGCCTGGATGGTC-3' and 5'-TGGAGGGCCACTGGATGAAG-3'. PCR products were purified and cloned into *Escherichia coli* (Invitrogen, Karlsruhe, Germany, TOPO10 cloning system) using the manufacturer's protocol.

Labeling of RNA probes

The digoxigenin-labeled RNA probes for murine ChAT (pAD1) were synthesized by using 2 µg of linearized template cDNA, 2 µl of T7 or Sp6 polymerase (1 U/µl, Maxiscript Sp6/T7 kit, Ambion, Cambridgeshire, UK), 0.7 µl of UTP 0.7 mM, 1 µl each ATP, GTP and CTP (1 mM each), 1 µl of digoxigenin-11-UTP 0.3 mM (Roche Diagnostics, Rotkreuz, Switzerland), 2 µl of 10× transcription buffer (Maxiscript Sp6/T7 kit, Ambion) and 1 µl of RNase Inhibitor (1 U/µl, Roche Diagnostics). Diethyl pyrocarbonate (DEPC)-treated H₂O (DEPC, Sigma, Munich, Germany) was added to a final volume of 20 µl.

The biotin-labeled RNA probes for murine NR2B (pMK4; Klein *et al.* 1998) and NR2C (pMK5; Pieri *et al.* 1999) were synthesized by using 2 µg of linearized template cDNA, 2 µl of T3, Sp6 or T7 polymerase (1 U/µl), 5 µl of Biotin-nucleotide mix (Perkin-Elmer, Rodgau – Jügesheim, Germany), 2 µl of 10× transcription buffer, 1 µl of RNase Inhibitor (1 U/µl) in a total volume of 20 µl and incubated for 2 h at 37 °C. The RNA probes were purified by LiCl/ethanol precipitation. The pellet was dried and resuspended in 50 µl of RNase-free H₂O and stored at –80 °C until use.

In situ hybridization

The animals were killed by cervical dislocation (Sethy & Francis 1988), their brains were quickly removed and 12-µm sections of wildtype C57BL/6J or NR2B-2C brain mice were cut using a cryostat (Leica, Solms, Germany). Sections were mounted on poly-L-lysine-treated slides (Polysine Slides,

OmniLabo, Artselaar, Belgium) and stored at -80°C before use. Sections were warmed to room temperature and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), at 4°C , for 30 min, washed in PBS for 1 min, incubated in HCl (0.6%) for 10 min, in Triton (PBS, 1% Triton-X-100) for 2 min. Sections were washed twice in PBS for 30 seconds and incubated in 50% formamide and $5\times$ SSC for 15 min.

The digoxigenin- or biotin-labeled RNA probes (250–400 ng/ml) were denatured in hybridization solution, at 65°C , for 5 min and subsequently chilled on ice for 5 min. The hybridization mix contains $0.2\ \mu\text{g}/\mu\text{l}$ of yeast tRNA (Roche Diagnostics), 10% dextran sulfate, $2\times$ SSC, $0.2\ \mu\text{g}/\mu\text{l}$ of herring sperm DNA (Sigma) and 50% formamide. One hundred microliters of denatured digoxigenin or biotin-labeled RNA probe in hybridization solution was applied to each section, and the hybridization was carried out at 55°C , for 18–24 h.

Sections were washed twice in $0.1\times$ SSC, at 60°C , for 30 min each time. Sections were treated with blocking solution [Tris-HCl 100 mM, pH 7.5, NaCl 150 mM, 0.3% Triton-X-100, 1% blocking solution (Roche Diagnostics)]. One hundred microliters of anti-digoxigenin-AP (1 : 500, Roche Diagnostics) and streptavidin-horseradish peroxidase (1 : 500) diluted in blocking solution (Roche Diagnostics) were applied to sections and incubated at 37°C , for 1 h, in a moist chamber. Sections were washed for three times with Tris-HCl 100 mM, pH 7.5, NaCl 150 mM and Tween-20 (0.5%), for 5 min.

To detect biotin-labeled probes, we applied the fluorophore tyramide (TSA Plus fluorescence systems, Perkin-Elmer) to slides for 15 min at room temperature. The detection of digoxigenin-labeled probes was performed by O/N incubation at 4°C with a Cy3-conjugated donkey anti-sheep IgG (Jackson, West Grove, PA, USA). Sections were washed for three times in Tris-HCl 100 mM, pH 7.5, NaCl 150 mM for 5 min and mounted in antifading solution (Slow-fade light plus Dapi kit, Molecular Probes, Karlsruhe, Germany). *In situ* hybridized sections were finally analyzed using an LSM 510 Zeiss laser scanning microscope.

Neurochemical analysis: brain ACh levels

In addition to the investigation of whether the wildtypes express the NR2C or NR2B protein in cholinergic neurons of the nucleus basalis, we also determined the ACh content in the frontal cortex and amygdala, the two target areas of the cholinergic projections of the nucleus basalis, 2 weeks after the end of behavioral testing in both genotypes. We further measured ACh levels in the hippocampus, which receives cholinergic input from the medial septum/vertical limb of the diagonal band, and in the striatum, where ACh is derived from intrinsic striatal neurons. The animals were killed by cervical dislocation (Sethy & Francis 1988), and their brains were quickly removed and placed in an ice-cold brain matrix. Coronal sections were made following landmarks on the base of the brain, and the frontal cortex, dorsal and ventral striatum, hippocampus and amygdala were dissected

out bilaterally onto an iced platform. Thereafter, the brain tissue was weighed, homogenized in ice-cold 0.05 N perchloric acid containing ethylhomocholine as an internal standard, centrifuged, filtered and kept at -70°C until being analyzed. The brain tissue samples were analyzed for ACh concentration according to the procedure utilized by Damsma *et al.* (1987), except for the internal standard (Potter *et al.* 1983), using high-performance liquid chromatography with electrochemical detection (for technical details, see De Souza Silva *et al.* 2000).

Behavioral analysis

Open field

In order to examine spatial novelty induced exploratory activity, behavioral habituation to a novel environment after a 24-h interval and recognition of spatial stimuli after a 72-h retention interval, we exposed the mice repeatedly to an open field. The open-field apparatus was a rectangular chamber ($29 \times 29 \times 40$ cm) made of gray Polyvinylchloride. A video camera was mounted 250 cm above the maze. Diffuse white light provided an illumination density of approximately 0.24 lux at the center of the maze. Masking noise was delivered by an active air conditioning system. The digitized image of the path taken by each animal was stored and analyzed with a semiautomated analysis system (EthoVision[®], Noldus, the Netherlands). After each trial, the apparatus was cleaned with water containing 0.1% acetic acid. We used a small open field, under low illumination conditions, in order to avoid interactions between learning and stress. Under more aversive conditions, such as a strongly illuminated large open space, the decrease in exploratory behaviors, normally observed during re-exposure to the open field, might reflect a memory for the experience of there being no escape route out of the field, rather than reflecting a simple form of spatial learning.

The behavioral parameters registered during 5-min sessions were (a) rearing – the number of times an animal was standing on its hind legs with forelegs in the air or against the wall was recorded manually using counters and (b) locomotion – the distance in centimeters an animal moved. The animals were given three trials in the open field, and the second and third trials were administered 24 and 96 h after the initial exposure.

Object exploration

Novel object exploration, behavioral habituation to novel objects and object recognition were evaluated in a familiar open field (see above). The mice were first presented with two identical objects, made of transparent glass with a height of 12 cm and a base diameter of 4 cm, placed in a balanced fashion in the corners of the open field. The mice explored the objects for 2 consecutive days during 5-min sessions. Thereafter, on the third day, one object was replaced by a novel glass object with a different shape, and the animals were allowed to explore the familiar and novel object for 5 min. The corner in which the novel object was

placed was balanced within the groups. Because the objects were made of the same material, they could not be distinguished by olfactory cues during the object recognition test. The objects had sufficient weight to ensure that the mice could not displace them. After each trial, the apparatus and the objects were thoroughly cleaned with water containing 0.1% acetic acid in order to remove odor cues. The objects had no known ethological significance for the mice and had never been paired with a reinforcer. Pilot studies ensured that C57BL/6 mice could discriminate the two objects, and there was no *per se* preference for one of these objects. Because the novel object differed from the familiar object only in shape but not regarding height, material or color, this novelty discrimination task was assumed to be rather difficult, having a high sensitivity for detecting promnesic effects. Additionally, we used rather long 24-h retention intervals to further increase the sensitivity of the task to ensure the detection of promnesic effects in NR2C-2B mice. For each mouse, the time spent exploring the objects was scored off-line from videotapes using stopwatches. The exploration of an object was assumed when the mouse approached an object and had physical contact with it, with its vibrissae, snout or forepaws. Vicinity to an object at a distance less than 2 cm was not considered as exploratory behavior.

Elevated plus maze

In order to probe whether unconditioned fear was affected by the NR2C-2B subunit exchange, we subjected the animals to the elevated plus maze. The plus maze consisted of two open arms (29 × 5 cm) and two walled arms (29 × 5 × 15 cm) with an open roof, arranged around a central platform (5 × 5 cm), in a way that the two arms of each type were opposite to each other. The maze was elevated to a height of 40 cm. The videotaped, digitized tracking, masking noise and illumination settings were the same as for the open-field experiment. After each trial, the apparatus was swept out with water containing 0.1% acetic acid. The mice were placed on the central platform of the maze facing one of the walled arms and were observed for 5 min, during which the number of entries into and time spent in the open and enclosed arms were measured.

Statistical analysis

Behavioral data were analyzed with repeated measures one-way ANOVAs and *t*-tests for dependent and independent samples. Neurochemical data were analyzed using *t*-tests for independent samples. All *P*-values given are two-tailed and were considered to be significant when *P* < 0.05.

Results

Mutant NR2B gene expression in cholinergic neurons of the nucleus basalis

To our knowledge, the expression of NMDA-R subunits in the nucleus basalis has been investigated neither in rats nor

in mice. It is unclear whether NMDA-R in the nucleus basalis expresses the NR2C and/or the NR2B subunits. Furthermore, it is not known whether these subunits are expressed in cholinergic cells of the nucleus basalis. We therefore performed double *in situ* hybridization using fluorescence-labeled probes against ChAT and NR2C or NR2B, respectively, to know whether (a) these subunits are expressed in the nucleus basalis of wildtype mice and (b) they are expressed in ChAT-positive cholinergic cells. Indeed, both NR2C and NR2B proteins are expressed in the nucleus basalis of wildtype mice (Fig. 1a,d) and are observed in ChAT-positive cells (Fig. 1c,f). Thus, we confirmed that NMDA-Rs on cholinergic cells of the nucleus basalis express both the NR2C and NR2B subunits in wildtype mice. In NR2C-2B subunit exchange mice, the coding region of the NR2C gene was replaced by a cDNA fragment coding for the NR2B protein, placed directly behind the ATG codon of the NR2C gene. Thus, these mice no longer express the NR2C protein in the brain. In adult wildtype mice, the highest level of NR2C protein expression is found in the cerebellum. Previously, we showed by means of Western blot analysis

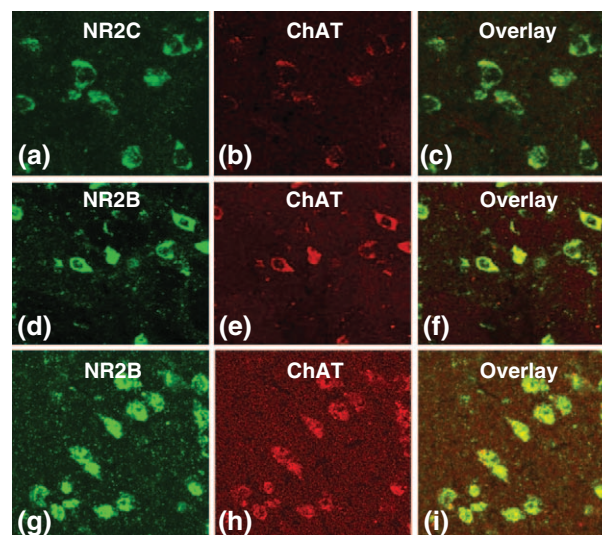


Figure 1: *In situ* hybridization for NR2C subunit and NR2B subunit mRNA in cholinergic neurons in the nucleus basalis.

(a) NR2C subunit mRNA antisense probe (green) in nucleus basalis of wildtype mice (×100). (b) Choline acetyl transferase (ChAT) mRNA antisense probe (red) in nucleus basalis of wildtype mice (×100). (c) Overlay shows the colocalization of NR2C subunit with ChAT in cholinergic neurons of nucleus basalis of wildtype mice. (d) NR2B subunit mRNA antisense probe (green) in nucleus basalis of wildtype mice (×100). (e) ChAT mRNA antisense probe (red) in nucleus basalis of wildtype mice (×100). (f) Overlay shows the colocalization of NR2B subunit with ChAT in cholinergic neurons of nucleus basalis of wildtype mice. (g) NR2B subunit mRNA antisense probe (green) in nucleus basalis of NR2C-2B mice (×100). (h) ChAT mRNA antisense probe (red) in nucleus basalis of NR2C-2B mice (×100). (i) Overlay shows the colocalization of NR2B subunit with ChAT in cholinergic neurons of nucleus basalis of NR2C-2B mice.

that the NR2C protein is absent in the cerebellar tissue of NR2C-2B subunit exchange mice (Schlett *et al.* 2004). Therefore, one can be certain that the NR2C protein is also absent in the nucleus basalis of NR2C-2B subunit exchange mice and that cholinergic cells which express the NR2C protein in the wildtypes are instead expressing the NR2B protein in the NR2C-2B subunit exchange mice. The coexpression of NR2B together with ChAT in the same cells was also confirmed for the NR2C-2B subunit exchange mice (Fig. 1g–i).

Neurochemistry: brain ACh levels

The *in situ* hybridization studies confirmed that murine cholinergic cells in the nucleus basalis express both the NR2C and NR2B subunits. The nucleus basalis cholinergic cells of NR2C-2B subunit exchange mice no longer express the NR2C protein but instead express additional NR2B subunits. We next evaluated whether this subunit exchange of nucleus basalis NMDA-R has an effect on ACh concentrations in the two main target areas, the frontal cortex and amygdala, which are innervated by these cholinergic cells (Luiten *et al.* 1985). Compared with wildtype littermates, the ACh levels of NR2C-2B mice were elevated by 27.6–21.8% in the frontal cortex ($P = 0.03$; Table 1, *t*-test for independent samples) and by 12.7–11.1% in the amygdala ($P = 0.03$; Table 1, *t*-test for independent samples). Our results suggest that the replacement of the NR2C by the NR2B subunit in nucleus basalis cholinergic neurons of NR2C-2B mutant mice increases ACh levels in the frontal cortex and amygdala. The hippocampus also receives a dense cholinergic innervation, which, however, rises from cells located in the medial septum and the vertical limb of the diagonal band (McKinney *et al.* 1983). Conversely, the striatum itself contains intrinsic cholinergic interneurons (Phelps *et al.* 1985). Both the hippocampus (Rafiki *et al.* 2000) and striatum (Beas-Zarate *et al.* 2002) express the NR2C subunit and should be likewise affected by the NR2C-2B subunit exchange in the mutant mice. We therefore also measured ACh levels in the hippocampus and striatum. Surprisingly, no statistically relevant changes were found in the hippocampus or in the striatum in the mutant mice compared with the wildtype mice (each $P > 0.1$).

Behavioral analysis

Behavioral habituation to an open field

Both groups showed behavioral habituation to the open field, as indicated by an effect of exposures on rearing activity and

locomotion (NR2C-2B: rearings: $F_{2,18} = 19.132$; $P < 0.001$; wildtype: rearings: $F_{2,16} = 9911$; $P = 0.002$; NR2C-2B: locomotion: $F_{2,18} = 29.378$; $P < 0.001$; wildtype: locomotion: $F_{2,16} = 20.098$; $P < 0.001$; repeated measures ANOVA, Fig. 2a,b). There was no significant main effect of *genotype* or *genotype* \times *day* interaction (both P -values > 0.05).

Behavioral habituation to objects

Across the 2 days, the NR2C-2B mice showed significant behavioral habituation to objects as indicated by an effect of exposures on the time spent exploring two equal objects ($F_{1,9} = 8540$; $P = 0.017$; repeated measures ANOVA; Fig. 2c, left). Wildtype mice failed to show statistically significant behavioral habituation to objects ($F_{1,8} = 4311$; $P = 0.072$). However, there was no significant main effect of *genotype* or *genotype* \times *day* interaction (both P -values > 0.05).

Object recognition

The NR2C-2B mice spent significantly more time exploring a novel object relative to a familiar object, as indicated by an effect of *objects* ($F_{1,9} = 5.276$; $P = 0.047$; repeated measures ANOVA; Fig. 2c, right). Wildtype mice failed to prefer the novel to the familiar object ($F_{1,8} = 1.650$; $P = 0.235$). However, there was no significant main effect of *genotype* or *genotype* \times *object* interaction (both P -values > 0.05).

Elevated plus maze

Neither the time spent in nor the number of entries into the open and walled compartments of the maze was statistically different between groups (time spent in open arms: $P = 0.26$, walled arms: $P = 0.90$; entries into open arms: $P = 0.48$, walled arms: $P = 0.2$; *t*-test for independent variables; Fig. 2d,e).

Discussion

To analyze the behavioral and neurochemical effects of perpetuating the NMDA-R subunit composition *in vivo*, we generated mutant mice, in which the coding sequence of the *NR2C* gene was replaced by that of the *NR2B* gene, whereas the promoter sequence of the *NR2C* gene was kept functional. These mutants express the NR2B subunit instead of the NR2C subunit throughout the brain. We previously showed in mature cerebellar granule cells that the NR2C protein is absent in the mutants and that the subunit exchange yields functional NMDA-R with distinct properties (Schlett *et al.* 2004).

Table 1: Mean (\pm SEM) concentration (pmol/mg) of acetylcholine in respective brain areas for NR2C-2B and wildtype mice

	Frontal cortex	Ventral striatum	Neostriatum	Hippocampus	Amygdala
NR2C-2B	7.625 \pm 0.683*	19.668 \pm 2.793	20.118 \pm 0.499	6.671 \pm 0.261	12.05 \pm 0.465*
Wildtype	5.723 \pm 0.292	19.318 \pm 0.859	19.732 \pm 0.947	6.738 \pm 0.545	10.611 \pm 0.311

* $P < 0.05$; *t*-test.

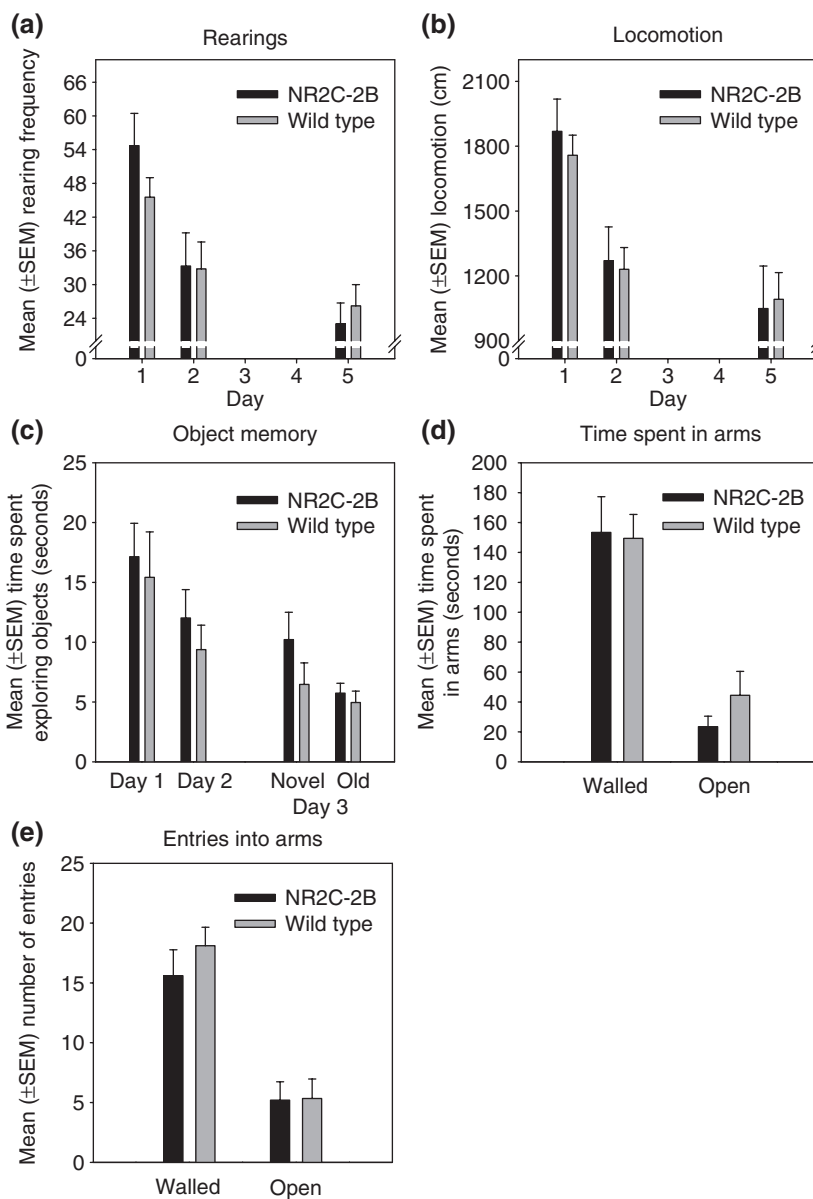


Figure 2: Behavioral analysis of NR2C-2B mutant and wildtype mice.

(a) Open field. Bars represent mean (\pm SEM) number of rearings on indicated days during 5-min sessions. (b) Open field. Bars represent mean (\pm SEM) locomotion in centimeters on indicated days during 5-min sessions. (c) Object memory. Effects of the NR2C-2B subunit gene replacement on habituation to object stimuli (day 2) and object recognition (day 3). Bars represent mean (\pm SEM) time spent exploring objects on indicated days. (d) Elevated plus maze. Bars represent mean (\pm SEM) time spent in seconds on indicated arms. (e) Elevated plus maze. Bars represent mean (\pm SEM) number of entries into indicated arms.

Cholinergic neurons in the nucleus basalis, from where cholinergic projections to the frontal cortex and basolateral amygdala arise, are excited by glutamate and blocked by NMDA-R antagonists but not α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA-R) antagonists (Fadel *et al.* 2001; Giovannini *et al.* 1997). The infusion of NMDA into the basal forebrain induces cortical ACh release (Fournier *et al.* 2004), and ACh-R activation enhances NMDA-mediated responses (Sabatino *et al.* 1999). Furthermore, basal forebrain NMDA-Rs contribute to the cortical ACh release after behavioral stimulation (Fadel *et al.* 2001). These studies suggest important functional interactions between glutamatergic and cholinergic systems at the level of the cholinergic basal forebrain.

Despite this evidence, suggesting a close functional link between glutamatergic and cholinergic systems, the subunit composition of NMDA-R located on nucleus basalis cholinergic cells in rodents is still unknown. Furthermore, it is unclear whether the NR2C or NR2B subunits are expressed in the nucleus basalis and whether these subunits are expressed in cholinergic neurons. We here demonstrate for the first time that both subunits are expressed in cholinergic cells of the nucleus basalis of mice. In light of these results, we next asked whether the changed NMDA-R subunit composition in nucleus basalis cholinergic neurons in NR2C-2B mice might have an impact on ACh content in the two main target areas of the cholinergic basal forebrain, e.g. the frontal cortex and amygdala. An HPLC-EC ACh assay revealed that the NR2C-

2B mice had indeed increased ACh levels in the frontal cortex and amygdala. These results suggest that the replacement of the NR2C by the NR2B subunit in nucleus basalis cholinergic neurons of NR2C-2B mutant mice affects cholinergic innervation of the frontal cortex and amygdala. This assumption is in accordance with the projection pattern of nucleus basalis cholinergic neurons (Luiten *et al.* 1985). The hippocampal formation expresses the NR2C subunit (Rafiki *et al.* 2000) and receives cholinergic input from cells located in the medial septum and the vertical limb of the diagonal band (McKinney *et al.* 1983). The NR2C subunit was also detected in the striatum (Beas-Zarate *et al.* 2002), which contains intrinsic cholinergic interneurons (Phelps *et al.* 1985). Because the mutant NR2C-2B mice express the NR2B subunit instead of the NR2C subunit in these structures, it can be assumed that ACh levels in the hippocampus and striatum should be likewise altered. Surprisingly, mutant mice did not differ from the wildtype regarding ACh levels in these brain areas. Thus, the subunit exchange has selectively affected ACh concentrations in the frontal cortex and amygdala.

Because the NR2C-2B subunit exchange is coupled to the developmental expression profile of NR2C, which is expressed postnatally and reaches adult levels within the fourth week after birth (Schlett *et al.* 2004; Sircar *et al.* 1996), developmental effects of the gene substitution might not account for these findings. However, although such results can be interpreted as an increase in release, synthesis or increase in the number of cholinergic synaptic terminals in these brain areas, it should be noted that these changes in post-mortem ACh content might also reflect changes such as metabolism and re-uptake (Finlay & Zigmond 1995). Therefore, it remains to be determined whether the increased frontal cortex and amygdala ACh levels are associated with changes in basal and novelty-induced ACh release. Furthermore, it remains to be determined whether NR2C and NR2B are expressed in the same or separate cholinergic cells. Regarding the first alternative, it would be also interesting to know whether these two subunits are found in the same NMDA-Rs or in different NMDA-R populations. NMDA-Rs containing both the NR2B and NR2C subunits have not yet been reported to occur in the rodent brain. The effect of NR2C-NR2B subunit replacement on NMDA-mediated responses in the nucleus basalis remains to be examined. Finally, by evaluating ACh levels in the frontal cortex and amygdala of NR2C-knockout mice (Kadotani *et al.* 1996), one can decide whether the present effects are due to the absence of the NR2C subunit or due to NR2B expression from the mutant *NR2C* gene locus.

It is known that NMDA-Rs are involved in cortical ACh release in brain structures relevant for learning and memory performance (Fadel *et al.* 2001; Fournier *et al.* 2004) and that the enhancement of cholinergic transmission in the brain can have promnesic effects (Sarter & Bruno 1997). ACh release in the frontal cortex was implicated in novelty-induced arousal, attention and the encoding of novel stimuli and

modulates memory consolidation (Acquas *et al.* 1998; Sarter & Bruno 2000). Cortical ACh regulates the gating or excitability of sensory neurons during the processing of novel stimuli. Increased ACh levels in the frontal cortex and amygdala might improve learning and memory performance by increasing attention to task-relevant stimuli (Cangioli *et al.* 2002; De Souza Silva *et al.* 2002; Giovannini *et al.* 2001; Pallares *et al.* 1998). When rats are exposed to a novel environment, they typically show exploratory behaviors concomitant with large increases of extracellular levels of cortical ACh. Both exploratory behaviors and extracellular levels of cortical ACh progressively decline when the environment becomes more and more familiar (Giovannini *et al.* 1998; Inglis *et al.* 1994). Object exploration and recognition also depends on an intact cholinergic innervation of cortical areas by the basal forebrain (Abe & Iwasaki 2001). It is known that the amygdala modulates the consolidation of the memories of emotionally arousing experiences (McGaugh 2004). The activation of ACh-Rs in the basolateral amygdala plays an important role in memory consolidation (Power *et al.* 2003). Furthermore, neuronal nicotinic ACh-Rs are permeable to Ca^{2+} and may interact with NMDA-R to increase intracellular Ca^{2+} concentrations required for cellular memory consolidation (Broide & Leslie 1999). Therefore, we subjected NR2C-2B mutant and wildtype mice to behavioral tasks related to encoding and retention of spatial and object novelty.

The decrease in exploratory behaviors over repeated exposures to a novel environment reflects a basic form of spatial learning (Cerbone & Sadile 1994). We therefore examined whether the NR2C-2B mice might show changes in behavioral habituation to novel spatial and object stimuli as well as object recognition after long retention intervals. However, open-field behavior of the NR2C-2B mutants was not significantly different from their wildtype controls.

The object recognition task bases on the principle that rodents spontaneously explore novel and familiar objects (Ennaceur & Delacour 1988) and tend to spend more time exploring a novel object relative to a familiar one. It is also possible to measure behavioral habituation to novel objects over multiple trials (Dere *et al.* 2003b). We used long 24-h retention intervals in order to reveal a possible promnesic effect of the NR2C-NR2B subunit replacement. However, neither the behavioral habituation to novel objects (days 1 and 2) nor object recognition performance was significantly different between genotypes. Nevertheless, within-subject analysis revealed that, in contrast to mutant mice, the wildtype mice failed to show significant behavioral habituation to novel objects and novel object recognition.

It has been argued that spatial or object novelty might represent a stressful condition and that the cholinergic activation after exposure to novelty is partly attributable to stress or emotionality. Furthermore, subunits of the NMDA-R have been implicated in unconditioned anxiety. NR2D-knockout mice showed reduced levels of anxiety (Miyamoto *et al.* 2002). Ifenprodil, a selective antagonist at NMDA-R featuring

the NR2B subunit, has been reported to induce anxiolysis in an elevated plus maze (Fraser *et al.* 1996), but not in the graded anxiety test (Dere *et al.* 2003a). The elevated plus-maze behavior of NR2C-2B mice was not statistically different from the controls, suggesting that the increased ACh concentrations in the amygdala of mutant mice had no effect on emotional reactivity to novel environments. These results suggest that despite inducing increased ACh concentrations in the frontal cortex and amygdala, the NR2C-2B subunit exchange has not affected anxiety-related behavior and behavioral habituation to spatial and object novelty, as well as object recognition. Nevertheless, it remains to be determined whether shorter or longer retention intervals and whether running the elevated plus-maze test under more aversive conditions, such as higher levels of illumination, would yield different results. It is also possible that the failure to observe behavioral effects, related to increased ACh contents in the frontal cortex and amygdala, might be due to compensatory changes in postsynaptic muscarinic and/or nicotinic ACh-R in terms of quantity and/or sensitivity.

Unlike our mutant mice, those in which the NR2B subunit was overexpressed in the forebrain displayed improved object recognition after long retention intervals (up to 3 days). The authors concluded that the increased expression of NR2B subunits in the hippocampus led to better learning and memory performance because of eased long-term potentiation (LTP) induction (Tang *et al.* 1999). It would be interesting to evaluate these mice in terms of frontal cortex and amygdaloid ACh content in order to know whether NR2B overexpression in these mice goes along with changes in brain cholinergic systems.

In conclusion, we showed, for the first time, that nucleus basalis cholinergic neurons express the NR2B and NR2C subunits. Furthermore, in NR2C-2B subunit exchange mice, ACh levels in the frontal cortex and amygdala were increased. It is possible that the replacement of the NR2C by the NR2B subunit in cholinergic neurons of the nucleus basalis led to this increased ACh content in two of its main target areas. However, despite the changes in ACh levels, behavioral habituation to novel environments and objects as well as object recognition after long retention intervals was unchanged after NR2C-2B subunit gene replacement.

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