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Maturation and maintenance of cholinergic medial septum neurons require glucocorticoid receptor signaling

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Summary
Glucocorticoids have been shown to influence trophic processes in the nervous system. In particular, they seem to be important for the development of cholinergic neurons in various brain regions. Here, we applied a genetic approach to investigate the role of the glucocorticoid receptor (GR) on the maturation and maintenance of cholinergic medial septal neurons between P15 and one year of age by using a mouse model carrying a CNS-specific conditional inactivation of the GR gene (GR^{NesCre}). The number of choline acetyltransferase and $p75^{NTR}$ immuno-positive neurons in the medial septum (MS) was analyzed by stereology in controls versus mutants. In addition, cholinergic fiber density, acetylcholine release and cholinergic key enzyme activity of these neurons were determined in the hippocampus. We found that in GR^{NesCre} animals the number of medial septal cholinergic neurons was significantly reduced during development. In addition, cholinergic cell number further decreased with aging in these mutants. The functional GR gene is therefore required for the proper maturation and maintenance of medial septal cholinergic neurons. However, the loss of cholinergic neurons in the medial septum is not accompanied by a loss of functional cholinergic parameters of these neurons in their target region, the hippocampus. This pinpoints to plasticity of the septo-hippocampal system, that seems to compensate for the septal cell loss by sprouting of the remaining neurons.
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Adrenal steroids mediate a plethora of physiological responses in both the periphery and CNS. Glucocorticoids, in particular, are known to play a crucial role in the development of various tissues and organs (Cole *et al*. 1993; Cole *et al*. 1995; Reichardt *et al*. 1998; Tronche *et al*. 1998; Bauer *et al*. 1999; Finotto *et al*. 1999; Reichardt *et al*. 2001; Gesina *et al*. 2004) including the CNS (Gould *et al*. 1991a; Gould *et al*. 1991b; McEwen 1999; Bakker *et al*. 2001; Demir and Demir 2001; Leret *et al*. 2004). Several studies have shown that stress which results in high plasma levels of corticosterone (CORT) or pharmacological application of synthetic glucocorticoids like dexamethasone (DEX) have an effect on trophic processes in the CNS and influence the maturation of cholinergic neurons during pre- and post-natal development. It is clear that pre-natal administration of DEX accelerates the maturation of cholinergic neurons in the retina (Puro 1983) and that early post-natal DEX treatment increases the synthesis of acetylcholine (ACh) in superior cervical ganglia (Sze *et al*. 1983). However, controversial findings have been reported on the development of the cholinergic septo-hippocampal projections upon depletion or administration of glucocorticoids. Takahashi and Goh (1998) found that maternal adrenalectomy delayed the appearance of

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Abbreviations used: ACh, acetylcholine; AChE, acetylcholine-esterase; ChAT, choline acetyltransferase; CORT, corticosterone; DEX, dexamethasone; FFT, fimbria-fornix transection; GR, glucocorticoid receptor; KH, Krebs-Henseleit; MR, mineralocorticoid receptor; MS, medial septum/diagonal band; NGF, nerve growth factor; OF, optical disector/fractionator method; PB, phosphate buffer.

the septo-hippocampal projections in P10 offsprings. This effect can be reversed by exogenous corticosterone application. Adrenalectomy at P10 leads to a reduced acetylcholinesterase (AChE) labelling density in the hippocampus at day P14 and this reduction can be prevented by the administration of exogenous CORT (Takahashi 1998). In contrast, Hu *et al*. (1996) found no effect of early post-natal DEX administration on cholinergic neurons in the MS but described an inhibitory effect of DEX on cholinergic development in other brain regions such as the caudate-putamen and diagonal band.

The cholinergic septo-hippocampal projection might be an important target for stress during development, leading to lifelong consequences on emotional behaviours as suggested by several studies (Day *et al*. 1998, Takahashi 1998). Long-term effects were observed both after pre- and post-natal interventions. Day *et al*. (1998). found that pre-natal stress increases post-natal hippocampal acetylcholine release in adult rat offsprings. Post-natal application of pharmacological doses of DEX at P7 increased both the number and the staining intensity of choline acetyltransferase or $p75^{NTR}$ immunopositive neurons in the medial septum. This effect was most likely mediated by an elevated level of NGF (Shi *et al*. 1998).

According to the cholinergic hypothesis of Alzheimer's disease (Perry *et al*. 1978; Bartus 2000) alterations of cholinergic nuclei in the basal forebrain account at least in part for cognitive deficits as observed during aging or in Alzheimer's disease. This hypothesis is supported by data for aged animals (Fischer *et al*. 1989) and experimental data from selective cholinergic lesions which resulted in memory deficits, although these impairments were not as dramatic as it was predicted by the cholinergic hypothesis (Parent and Baxter 2004).

Glucocorticoids are known to be involved in cognitive decline during aging (Lupien *et al*. 1995; Belanoff *et al*. 2001). It has been observed that excessive circulatory levels of endogenous corticosteroids as well as exogenous delivery of corticosteroids are frequently associated with cognitive impairment in a wide variety of clinical disease states. Cognition and low levels of corticosteroids have been less well studied (Belanoff *et al*. 2001). However, a relative glucocorticoid resistance has been described in brains of patients suffering from Alzheimer's disease (Dai *et al*. 2004).

In the present study, we used a genetic approach in mice to investigate the role of GR in the development of cholinergic septo-hippocampal neurons and in their maintenance during adulthood. To this end we compared control animals ($GR^{loxP/loxP}$) at P15, 3.5 months and one year of age with brain-specific conditional GR mutant mice ($GR^{loxP/loxP}$; Tg:NesCre, designed GR^{NesCre} in this study, Tronche *et al*. 1999). This animal model has been developed using the Cre/*loxP* system. GR inactivation is driven by the Cre recombinase expressed under the control of a construct containing the rat nestin gene promoter and enhancer. In this model, recombination should occur early around E10 depriving neuronal and glial cells of GR protein during the whole period of medial septum development, i.e. from the last third of gestation until adulthood (Bayer 1979; Naumann *et al*. 2002). By quantitative stereology we determined the number of cholinergic MS neurons in GR^{NesCre} mice and control litter-mates. In addition, cholinergic fiber density and functional parameters of cholinergic terminals such as choline acetyltransferase (ChAT) activity and acetylcholine (ACh) release were determined in the hippocampus at the same time points.

We found that at 3.5 months of age the number of ChAT- and $p75^{NTR}$-immunopositive cholinergic neurons in the MS was significantly reduced in brain-specific GR mutants. Hippocampal cholinergic fiber density and functional parameters of cholinergic terminals remained however, unchanged to that of control litter-mates. These results show that GR gene is essential for a correct development of septal cholinergic neurons, strongly suggesting that basal levels of CORT are required for this. However, the reduction in cholinergic septo-hippocampal neurons during development seems to be compensated for by an up-regulation of cholinergic key enzymes and sprouting of the remaining neurons. In addition to this early effect in one year old mutant animals we observed a further decrease in the total number of cholinergic MS neurons, indicating a further role for GR gene in the survival of these neurons.

Materials and methods

Animals

Generation of conditional, brain specific GR mutant mice (GR^{NesCre}) was described in detail elsewhere (Tronche *et al*. 1999). Briefly, GR gene is inactivated by the Cre-mediated excision of the third exon. In GR^{NesCre} mice, Cre recombinase expression starting at E10 is mostly restricted to neuronal and glial precursor cells leading to a complete GR gene inactivation in almost all neurons and glial cells of the CNS. Mice were housed under a 12 h-12 h dark light cycle and fed ad libitum. Due to the mutation GR^{NesCre} animals have higher CORT levels that can not exert their effects through the GR in the brain (Tronche *et al*. 1999). We previously excluded the establishment of compensatory mechanisms that would rely on an over-expression of the type I or mineralocorticoid gene that encodes for the second nuclear CORT receptor (Gass *et al*. 2000). The following numbers of animals were used for the different experiments: Stereology/cell count ($n = 6$ per genotype and time point), AChE densitometry ($n = 6$ per genotype), volumetry of the hippocampus ($n = 2$ per genotype), fimbria-fornix transection ($n = 2$ per genotype), ChAT activity ($n = 6$ per genotype) and ACh release ($n = 6$ per genotype).

Tissue processing for histochemistry and immunocytochemistry

Mice were transcardially perfused first with 0.9% saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.35). Whole brains were post-fixed in the same fixative for about 2 h. Coronal 50 μm sections were cut on a vibratome across

the entire septal region and collected to reconstruct the complete series. To assess the density of cholinergic innervation, serial hippocampal sections of all animals were processed for AChE histochemistry using a modified Karnovsky and Roots protocol (Mesulam *et al*. 1987). ChAT immunocytochemistry was performed with a goat anti-ChAT polyclonal antibody (1 : 500 in 0.1 M PB containing 5% normal rabbit serum and 0.5% Triton X-100; Bioproducts, Boehringer Ingelheim, Ingelheim, Germany) for 48 h at 4°C, followed by biotinylated rabbit anti-Goat IgG. $p75^{NTR}$ immunocytochemistry was performed using an antihuman antibody (catalog #G3231; Promega, Madison, WI, USA) diluted 1 : 1000 in 0.1 M PB containing 1% normal goat serum and 0.5% Triton X-100. Immunostaining was visualized using the avidin-biotin complex (Elite Kit, Vector Laboratories, Burlingame, CA, USA) followed by DAB reaction. Every second section of each complete series was used for statistical analysis. Sections were mounted on slides, dehydrated, and coverslipped using Histokit (Shandon, Pittsburgh, PA, USA).

Quantification of AChE staining

The density of cholinergic fibers in subregions of the hippocampus was determined by densitometry ($n = 6$ per genotype). Using computer-assisted image analysis (SIS, Stuttgart, Germany), the mean optical density (OD) was determined on digitalized images after delineation of regions of interest, i.e. the CA1, CA3 subregions and the dentate gyrus, both in the ventral and dorsal hippocampus. The mean OD determined in the region of the corpus callosum in each section was considered as 'background' and subtracted from all mean ODs measured in the regions of interest.

In order to test the validity of the determined densitometrical data we performed volume measurements of the hippocampus in control and GR^{nescre} mice using the Stereo Investigator (version 3.0; MicroBrightField, Inc., Colchester, VT, USA). We performed bilateral volume measurements of the hippocampus in two animals per genotype.

Fimbria-fornix transection

In order to test our hypothesis that the cholinergic fibers in the hippocampus, especially the unaltered fiber density in the mutants, is due to sprouting of the remaining cholinergic medial septal neurons, we performed fimbria-fornix transection in two adult animals per genotype (for detailed description see Naumann *et al*. 1992). Briefly, adult female control and GR^{Nescre} mice were anesthetized with a mixture of ketamine, rompun and ketavet (2.5 mL/kg body weight, i.p) and placed in a stereotaxic apparatus. The skull was opened bilaterally 1 mm posterior to bregma, extending 5 mm on either side of the midline. Under visual control, the overlying cortical tissue was removed by aspiration to access the fimbria-fornix, which was aspirated bilaterally. All surgery was performed in accordance with institutional guidelines for animal welfare. The animals were allowed to survive for 14 days.

Cell counts: stereology

The number of cholinergic, MS neurons, i.e. ChAT- and $p75^{NTR}$-immunopositive neurons, was estimated by means of the optical disector/fractionator method (OF; see West *et al*. 1991). For this we have first determined the 'region of interest', the medial septal nucleus, in the control animals and the corresponding GR^{NesCre} mutants ($n = 6$ per genotype and time point) by combined retrograde tracing and immunocytochemistry for ChAT and $p75^{NTR}$.

The anatomical boundaries used to define the basal forebrain nuclei were already described in detail (see: Peterson *et al*. 1999), however, for estimation of cell numbers in the medial septum we modified this approach in line with our results obtained after retrograde labeling of septo-hippocampal MS neurons (see Naumann *et al*. 2002).

We were unable to detect any difference regarding the distribution of the septo-hippocampal projection neurons and their staining for the two cholinergic marker proteins (data not shown) when compared with our previous data obtained from mice of the same genetic background (cf. Naumann *et al*. 2002; Naumann *et al*. 2003). In principle, the position of the septo-hippocampal projection neurons along the rostro-caudal axis of the brain was not affected in the glucocorticoid receptor mutant mice (data not shown).

For stereology, sections of the septal region were visualized on a computer screen attached to an Olympus BX60 microscope F5 (Olympus Optical Co. Ltd, Düsseldorf, Germany). A computer-controlled stepper motor stage and focus assembly allowed movement in the *x*-, *y*- and *z*-axes. Cell counts were performed using Stereo Investigator software (version 3.0; MicroBrightField, Inc., Colchester, USA). The region of interest (Figs 1a and b) was first marked for every single section using low-power magnification (4x/0.10 objective). For subsequent cell counts, the following parameters were added to the program: counting frame, 50×30 μm; guard zone, 2 μm; and counting depth, 8 μm. Thereafter, using high-power magnification (oil objective lens, 100x/1.35), ChAT- and $p75^{NTR}$-positive cells that fulfilled the criteria of the unbiased counting rules (e.g. presence of the recognizable soma meeting the counting frame, somata showing a distinct nucleolus; cf. Coggeshall and Lekan 1996) were marked and added to the probe run list. Total cell numbers, estimated by the OF, were subsequently statistically analyzed by two-way-analysis of variance (ANOVA; for details see Naumann *et al*. 2002). Statistical significance was analyzed for the corresponding two classifers (ChAT, $p75^{NTR.}$) and two classes (control and GR^{Nescre} mutant mice).

Choline acetyltransferase (ChAT) activity

GR^{NesCre} and control litter-mates ($n = 6$ of each genotype) were killed by decapitation, their brains quickly removed and both hippocampi dissected. One hippocampus of each mouse was used for superfusion experiments (see below); the other one was homogenized in 1 mL 0.32 M sucrose (in 2.5 mM HEPES, pH 7.4) using a Potter Elvehjem (Braan-Melsungen, Melsungen, Germany) glass/Teflon homogenizer (8 strokes at 500 r.p.m). From this crude homogenate the following aliquots were prepared and stored at −80°C until measurement: a 20 μL sample (diluted with 180 μL 0.1 N NaOH) for determination of protein (see Lowry *et al*. 1951) and a 100 μL aliquot for determination of ChAT activity (see Fonnum 1975) with modifications. In brief, the 100 μL aliquot of the crude homogenate was diluted with 100 μL of a freshly prepared medium containing 0.32 M sucrose, 129 mM NaCl, 88 mM NaH_2PO_4, 2.5 mM HEPES, 0.9 mM EGTA, 0.9 mM Na_2EDTA, 179 μM physostigmine and 0.45% Triton-X100. Twelve microliters of this mixture (all samples in triplicates) were added to 6 μL of choline bromide (32 mM). The incubation was started by the addition of 6 μL of [^{14}C]acetyl-coenzyme-A (50 nCi/assay;

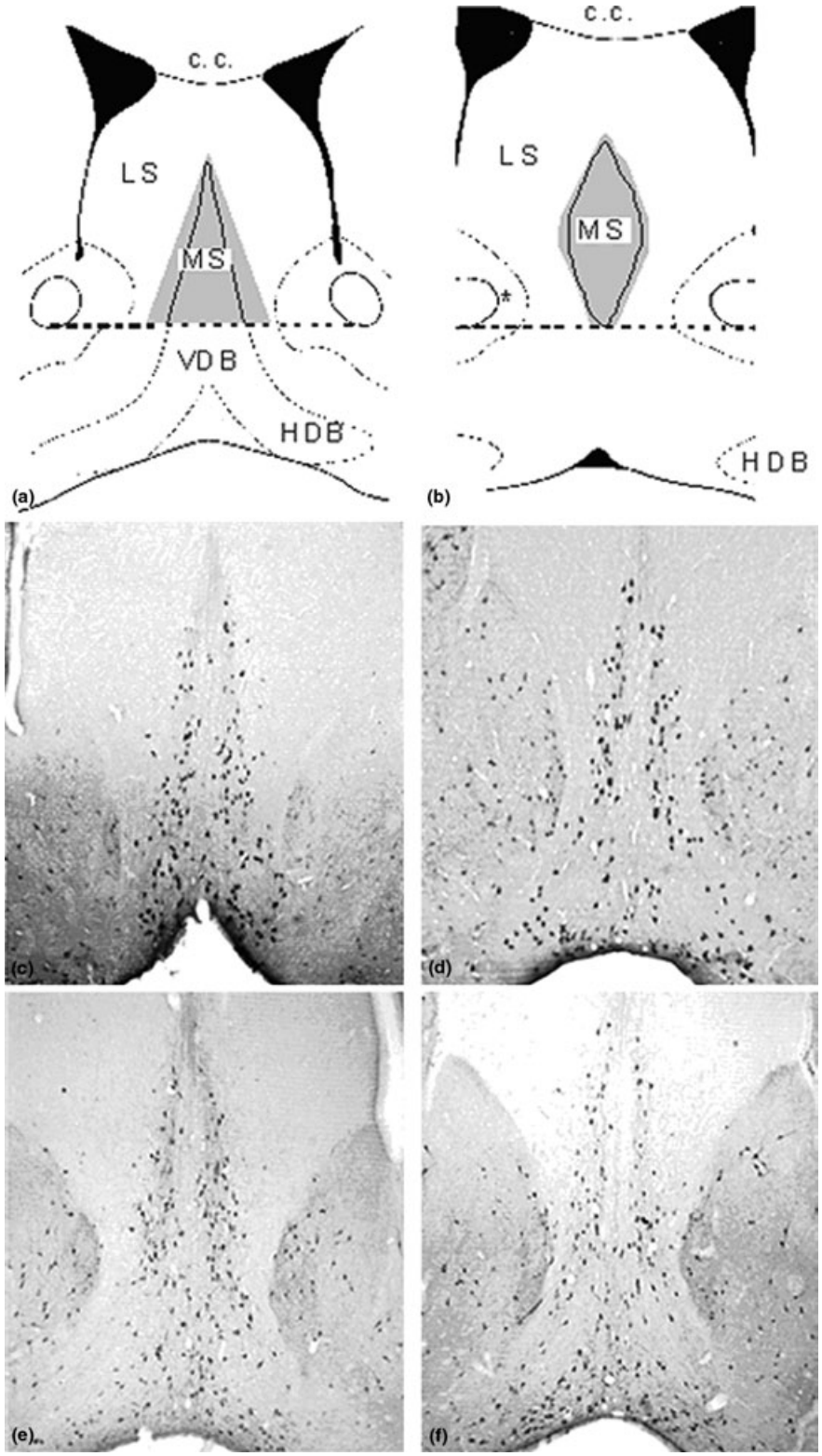

Fig. 1 Schematic drawing of the MS in a rostral (a) and caudal (b) section. The grey field shows the region of interest as it was outlined for stereological analysis. Distribution of ChAT–immunopositive MS neurons at the level of (a) in P15 (c, d) and 3.5 months (e, f) old control (c, e) and GR^{NesCre} mice (d, f). c.c. = corpus callosum, LS = lateral septum, MS = medial septum, VDB = vertical diagonal band, HDB = horizontal diagonal band.

0.227 mM final concentration) and vigorous mixing. After 20 min at 37°C, 20 microliters of the mixture were pipetted into a mixture of 5 mL sodium phosphate buffer (10 mM; pH 7.4) with 2 mL sodium tetraphenylborate in acetonitrile (5 mg/mL). From this mixture the newly formed [^{14}C]ACh was extracted by careful shaking with 10 mL of toluene scintillator. Following separation of the aqueous from the organic phase, the samples were directly counted by liquid scintillation counting. In order to correct for non-specific effects, for each homogenate two samples were run at 0°C. ChAT activity was calculated as nmoles ACh formed per min and per mg protein. For statistics the unpaired *t*-test was used ($N = 6$ mice per genotype).

Release of [^{3}H]ACh

The second hippocampus of each mouse ($n = 6$ per genotype) was cut into 300 μm thick slices using a McIlwain tissue chopper (Campden Instruments Ltd., Loughborough, England). The slices were pre-incubated for 45 min at 37°C under carbogen in 2 mL Krebs-Henseleit (KH) buffer containing [^{3}H]choline (0.1 μM). The KH solution had the following composition (in mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 10; ascorbic acid, 0.6; Na_2EDTA, 0.03; saturated with carbogen, pH adjusted to 7.4. Following pre-incubation of the slices, removal of the radioactive medium and several washing steps, they were transferred to superfusion chambers (12 chambers per superfusion apparatus, 1 slice per chamber) and superfused with oxygenated KH buffer (37°C) containing hemicholinium-3 (10 μM) at a rate of 1.2 mL/min. Fractions (2 min) to be measured were collected from 32 min of superfusion onwards. The overflow of [^{3}H]ACh was induced by three periods of electrical field stimulation (360 rectangular pulses at 3 Hz, 2 ms, 4 V/chamber, 26–30 mA) after 36 min (S_1), 52 min (S_2) and 68 min (S_3) of superfusion. Drugs to be tested, i.e. physostigmine, 1 μM (or physostigmine, 1 μM + atropine, 1 μM, respectively) were added to the superfusion medium of some chambers from 8 min before S_2 (or S_3, respectively) onwards. At the end of the experiment (after 76 min of superfusion) the radioactivity of superfusate samples and slices (dissolved in 250 μL Solvable, Packard, Frankfurt, Germany) was determined by liquid scintillation counting. The 'fractional rate of tritium outflow' (in percent of tissue tritium per 2 min) was calculated as: (pmoles tritium outflow per 2 min) × 100/(pmoles tritium in the slice at the start of the corresponding 2-min period). The 'baseline tritium outflow' (b_1) in the fraction preceding S_1 (i.e. from 34 to 36 min of superfusion) is given either in *absolute* terms (nCi [^{3}H]outflow) or in *relative* terms ('fractional rate of tritium outflow per 2 min'). The 'stimulation-evoked overflow of tritium' was calculated by subtraction of the baseline outflow and is shown either in *absolute* terms ('nCi' [^{3}H] overflow) or in *relative* terms (in per cent of the tritium content of the slice at the onset of the respective stimulation period). Effects of drugs added before S_2 and S_3 were determined as the ratio of the overflow evoked by the corresponding stimulation period (S_2/S_1 or S_3/S_1) and compared to the appropriate control ratio (no drug addition before S_2 and S_3). For statistical analysis, all data from superfused hippocampal slices of each individual mouse were first pooled for calculation of mean values. Only these mean values (N = number of mice per group) were then used for statistical comparison (ANOVA, or unpaired *t*-test, where appropriate).

All experiments were performed in accordance with the German and French laws on the use of laboratory animals.

Results

Number of cholinergic neurons in the MS

In order to address the role of the GR gene in controlling the number of cholinergic neurons within the septum, we compared GR^{NesCre} and control litter-mates. We stained vibratome serial coronal sections with antibodies directed against ChAT (Fig. 1c-f) and p75^{NTR} as these proteins are specifically expressed in cholinergic neurons. Strong labeling of cells was visible within the MS in control and mutant mice at post-natal day 15 (P15; Figs 1c and d) and at the age of 3.5 months (Fig. 1e and f). Although the number of cholinergic MS neurons was not significantly reduced in P15 mutant mice (Fig. 1c and d) a clear reduction was observed in 3.5 months old mutants (Fig. 1e and f). In order to precisely quantify the number of cholinergic cells in the control and mutant animals throughout development, we performed a stereological analysis on stained neurons. In control mice (Fig. 2, grey columns) the number of ChAT-immunoreactive MS neurons increased during development and reached a maximum of approximately 3500 at the age of 3.5 months. In contrast, the development of ChAT-immunoreactive cholinergic MS neurons was strongly reduced in mutant mice (Fig. 2, dark columns). The maximal number at 3.5 months was only 2080, a number significantly lower than in the controls ($p < 0.05$).

Because more than 90% of the cholinergic MS neurons co-express ChAT and p75^{NTR} (Sobreviela *et al*. 1994) we performed, as a control, the same stereological analysis for p75^{NTR} stained neurons in 3.5 months old animals. As expected, in 3.5 months old GR^{NesCre} mice and their control litter-mates the total number of p75^{NTR}-immunoreactive cholinergic MS neurons was slightly lower than the values

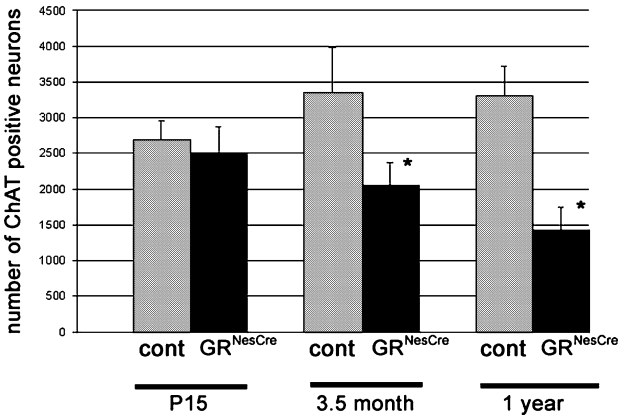

Fig. 2 Quantitative stereological analysis of ChAT immunopositive cholinergic MS neurons in control (cont) and GR mutant (GR^{NesCre}) mice at P15 and the age of 3.5 months and one year. GR mutants show a significant reduction in the number of ChAT positive neurons at 3.5 month (* $p < 0.05$) and one year ($p < 0.01$) of age. Means ± SD of $n = 6$ mice per time point and genotype.

observed for ChAT-immunoreactive neurons. However, the same significant decrease as in the number of ChAT positive MS neurons was found in the mutants for $p75^{NTR}$-immunoreactive neurons (data not shown). While at one year of age the number of ChAT positive neurons in controls remained approximately at the same level as observed at 3.5 months, the GR mutants showed a further decrease in cholinergic MS neurons. At one year of age they were reduced to an average of 1400 ChAT positive neurons, a number which is significantly lower than in control litter-mates (Fig. 2, $*p < 0.01$).

Density of cholinergic fibers in the hippocampus

Most of the medial septal cholinergic neurons project into the hippocampus. As we found an effect of GR gene mutation on the number of cholinergic cells in the septum, we wanted to find out whether this leads to measurable consequences in the target region of these cells. We therefore stained hippocampal sections for AChE, a specific marker for the cholinergic septo-hippocampal projections and performed a densitometrical analysis of the fiber density in subregions of the hippocampus. Surprisingly, no decrease in the density of cholinergic fibers in the mutant animals was detectable in any hippocampal subregion investigated (Fig. 3). In order to validate these densitometrical findings we performed volumetry of the control and GR^{nescre} mutants. We observed a mean unilateral hippocampus volume of 10.75 ± 0.81 mm^3 in the control and of 10.73 ± 0.21 mm^3 in GR^{nescre} mutants. These values are not significantly different between the genotypes. Thus, our densitometrical data on cholinergic fiber density are reliable.

Because various lesion experiments have clearly shown that the cholinergic input to the fascia dentata and the hippocampus proper in rodents derives from the MSDB complex (Naumann *et al*. 1992; Naumann *et al*. 1997), one possible explanation for this observation might be a compensatory sprouting of the remaining cholinergic medial septal neurons during the development of GR^{NesCre} mice. This view was supported by the results of the fimbria-fornix transection (FFT), lesioning the cholinergic septo-hippocampal projection. Two weeks after FFT the cholinergic fiber network in the hippocampus disappeared in both genotypes. Thus, the remaining cholinergic neurons in the medial septum of GR^{Nescre} mice have to be the origin of the cholinergic fiber network in the hippocampus.

Functional cholinergic parameters in the hippocampus

In order to also obtain *functional* parameters about the density of the cholinergic innervation in the target region of the MS, the hippocampus an *in vitro* brain slice superfusion technique was applied to determine various presynaptic parameters of cholinergic neurotransmission in this brain region and the enzymatic activity of ChAT was determined in hippocampal homogenates.

The electrically evoked overflow of [^{3}H] from mouse hippocampal slices pre-incubated with the tritiated precursor of ACh (i.e. [^{3}H]choline) was Ca^{2+}-dependent and sensitive to tetrodotoxin (data not shown). Hence, the evoked overflow of [^{3}H] from these slices represents a model for exocytotic, action potential-evoked release of [^{3}H]ACh. The time course of basal and electrically evoked release of ACh in hippocampal slices originating from control and GR mutant mice is shown in Fig. 4. It is evident, that the presence of the AChE inhibitor physostigmine (1 μM) during the second stimulation period (S_2) strongly inhibited the evoked release of [^{3}H]ACh. In the additional presence of the muscarine receptor antagonist atropine (1 μM) during the third stimulation period (S_3), the inhibitory effect of physostigmine was completely antagonized and even a significant facilitatory effect could be observed. The statistical evaluation of these effects is summarized in Fig 5(e and f): physostigmine inhibited the evoked release of ACh by about 60% ($p < 0.001$) whereas the additional presence of atropine facilitated the evoked release of ACh by about 40% ($p < 0.001$). Interestingly, however, no differences in the muscarine receptor mediated presynaptic modulation of ACh release between control and GR^{NesCre} mice, respectively, were detectable.

Also in Fig. 5, further parameters of cholinergic transmission in the hippocampus are shown. For instance, the accumulation of [^{3}H]choline by hippocampal slices (Fig. 5a) was not significantly different in control and GR^{NesCre} mice. Similarly, both the evoked release of [^{3}H]ACh (Fig. 5c) and the basal outflow of [^{3}H] (Fig. 5d) in hippocampal slices were unchanged by the GR mutation, regardless of whether they were expressed in *absolute* (i.e. in nCi) or *relative* amounts (i.e. in percentage of tissue-^{3}H).

Finally, as regards the activity of ChAT, Fig. 5(b) shows, that the activity of this enzyme in the hippocampus of GR mutant mice was almost identical to that in control mice.

Discussion

Glucocorticoids have been reported to play an important role in the development of several brain regions including the septal complex and its target region, the hippocampus (Gould *et al*. 1991a; Gould *et al*. 1991b; McEwen 1999; Kim and Diamond 2002). The maturation of cholinergic septal neurons, including their functional parameters, seems to be under the influence of glucocorticoids (Day *et al*. 1998; Shi *et al*. 1998; Takahashi 1998). However, these studies used either post-natal application of pharmacological doses of glucocorticoids or stress exposure which both lead to high plasma glucocorticoid levels. Moreover, some of the reported findings are contradictory concerning the effect of glucocorticoids on cholinergic maturation (Hu *et al*. 1996).

In the present study we used a genetical approach to investigate the role of glucocorticoid receptor gene on

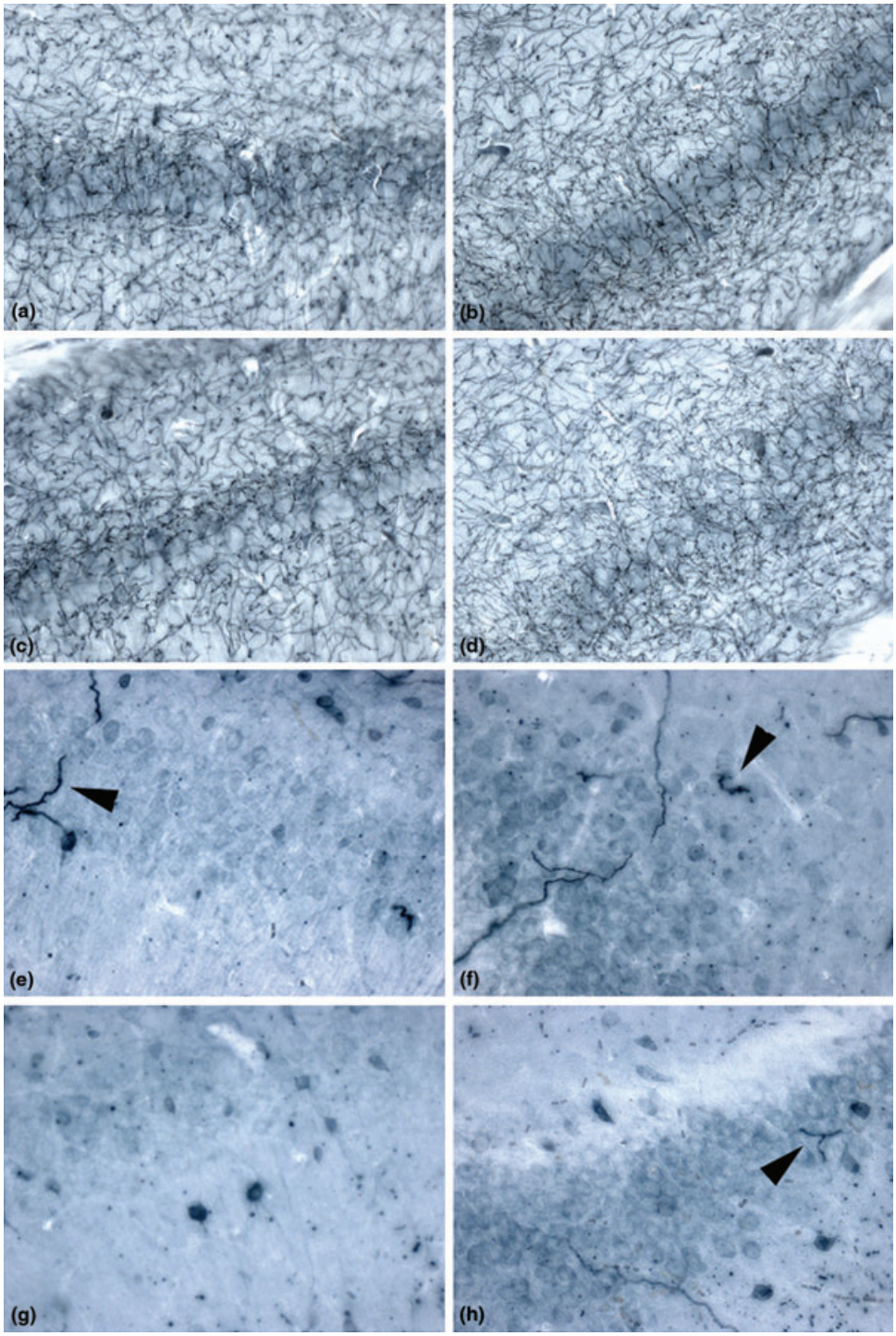

Fig. 3 Acetylcholine-esterase staining in hippocampal regions CA1 (a, c) and CA3 (b, d) of 3.5 months old control (a, b) and GR^{NesCre} mice (c, d). No difference in the density of cholinergic innervation of the hippocampus was found between genotypes. Two weeks after fimbria-fornix transection the cholinergic fiber network almost completely disappeared in both genotypes (e, f: CA1 and CA3 regions of lesioned control mice; g, h: CA1 and CA3 regions of lesioned GR^{nescre} mice). Only very few fragmented and swollen, degenerating cholinergic fibers (arrowheads) could be observed at that time point after lesioning the septo-hippocampal projection. Scale bar indicating 100 μm in a-d and 50 μm in e-h.

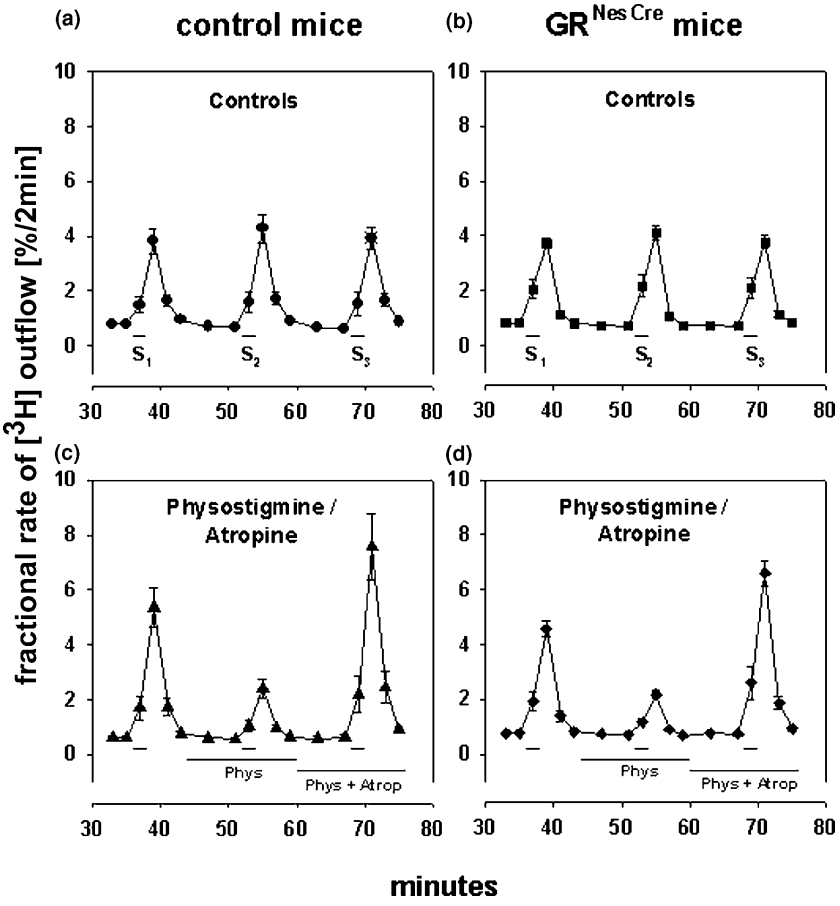

Fig. 4 Time course of tritium outflow in hippocampal slices from control (a, c) and GR^{NesCre} mice (b, d); effects of the AChE inhibitor physostigmine and the M receptor antagonist atropine. Following pre-incubation in the presence of [^{3}H]choline, the slices were superfused with physiological buffer (37°C) in the presence of 10 μM hemicholinium-3 at a rate of 1.2 mL/min. The fractional rate of tritium outflow in these slices is shown at the time indicated by the abscissa. During the time periods indicated by small horizontal bars (S_1, S_2, S_3) electrical field stimulation was applied (360 pulses, 3 Hz, 2 ms, 26–30 mA). Panels a and b show control experiments (no drug additions), whereas in panels e and f physostigmine (1 μM), or physostigmine + atropine (1 μM, each) were present during the second (S_2) and third (S_3) stimulation periods, respectively, as indicated by the large horizontal bars; means ± SEM of 6 hippocampal slices of either control or GR^{NesCre} mice ($n = 6$ per genotype).

cholinergic MS neurons. We analyzed the number of cholinergic MS neurons at different time points in mouse mutants carrying a conditional CNS specific mutation of the glucocorticoid receptor gene. The mutation takes place at E10, i.e. before the first known effect of glucocorticoids on MS development which begins at E13 (Lawson *et al*. 1977; Bayer 1979; Semba and Fibiger 1988) and lasts in mice until about P90 (Naumann *et al*. 2002).

A main observation of the present study is that the conditional CNS specific mutation of the GR receptor gene during neuronal development, strongly decreased the number of cholinergic neurons in the MS. This effect was due to the lack of GR signaling. A second receptor, called. 'type I' or mineralocorticoid receptor (MR), is expressed in restricted brain regions (Kretz *et al*. 2001) and can also bind glucocorticoids with a tenfold higher affinity than the GR. To exclude compensatory effects mediated via the MR, we examined in an earlier study the pattern and level of MR expression in the brain of GR^{NesCre} mutants. We found that MR is neither up-regulated at the mRNA nor at the protein level in brains of GR^{NesCre} mutants (Gass *et al*. 2000).

A second important observation is that the lack of GR protein in neurons and glial cells leads to an ongoing loss of ChAT-positive neurons resulting in a dramatically reduced cell number in one year-old GR^{NesCre} mice. In contrast to the reduction of cholinergic neurons in the MS of GR^{NesCre} mice, no modifications were observed in the hippocampus, the region innervated by these neurons. Both cholinergic fiber density and functional cholinergic parameters remained unaltered.

GR signaling and medial septal cholinergic neurons

Both the development and the survival of cholinergic MS neurons have been reported to be controlled by neurotrophic factors synthesized by cholinoceptive cells in the hippocampus (for review see Korsching *et al*. 1985; Sofroniew *et al*. 2001). In rats, hippocampal NGF synthesis strongly increases during the first post-natal weeks (Large *et al*. 1986). During the same period cholinergic septal neurons up-regulate the NGF receptors $p75^{NTR}$ and trkA (Large *et al*. 1986; Whittemore *et al*. 1986; Auburger *et al*. 1987; Cavicchioli *et al*. 1989; Roback *et al*. 1992; Ringstedt *et al*. 1993). The axonal uptake and retrograde transport of NGF then triggers the maturation of cholinergic MS neurons. For instance, blockade of NGF signaling has been shown to lead to a strongly reduced ChAT expression and thus to an impaired cholinergic maturation in the MS of rats (Hefti *et al*. 1985; Vantini *et al*. 1989; Svendsen *et al*. 1994). Similar results have been described for mice (Virgili *et al*. 1991). In aged rats and mice the septal expression of NGF receptor trkA is down-

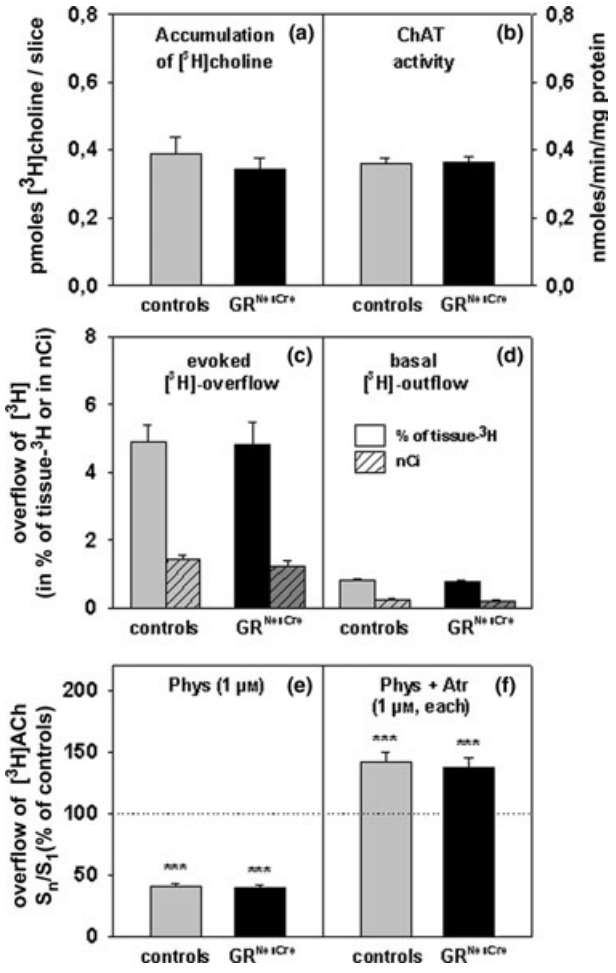

Fig. 5 Cholinergic parameters in hippocampal tissue of control and GR^{NesCre} mice. (a) accumulation of [^{3}H]choline into hippocampal slices (in pmoles/slice). (b) Specific activity of ChAT in homogenates of hippocampal tissue (in nmoles ACh formed/min/mg protein). (c) Electrically evoked overflow of [^{3}H] (which corresponds to ACh release) during the first stimulation period (S_1, see Fig. 4) in slices pre-incubated with [^{3}H]choline; results are shown as relative amounts (in percentage of tissue-^{3}H; open columns) or in absolute amounts (in nCi; hatched columns). (d) Basal outflow of [^{3}H] in slices pre-incubated with [^{3}H]choline; results are shown as relative amounts (in percentage of tissue-^{3}H; open columns) or in absolute amounts (in nCi; hatched columns). (e) Effects of physostigmine (1 µM) present during S_2 (see Fig. 4) on the evoked overflow of [^{3}H] in slices pre-incubated with [^{3}H]choline; results are shown as S_2/S_1 ratios expressed in percent of the corresponding control ratios. (f) Effects of physostigmine + atropine (1 µM, each) present during S_3 (see Fig. 4) on the evoked overflow of [^{3}H] in slices pre-incubated with [^{3}H]choline; results are shown as S_3/S_1 ratios expressed in percent of the corresponding control ratios. Data shown in panel a and panels c-f were obtained from 7 to 8 control and GR^{NesCre} mice (each), data in panel b were obtained from the hippocampi of 11 control and 8 GR^{NesCre} mice. Statistics: in all panels (a-f) no significant differences in between the corresponding values from control and GR^{NesCre} mice ($n = 6$ per genotype) were observed; panel e and f: ***$p < 0.001$ vs. corresponding control ratios (no drug addition before S_2 and S_3, respectively.

regulated and the retrograde transport of NGF is reduced (Cooper *et al*. 1994). As a result, aged rats and mice of about 1.5 years of age display a progressive atrophy of basal forebrain cholinergic neurons. Also in the human brain a progressive decrease of cholinergic transmission has been observed during aging (Feuerstein *et al*. 1992) and thus decreased cognitive functions in aging and dementia have been linked to the cholinergic system (Bartus *et al*. 1982; Hellweg *et al*. 1990; Alberch *et al*. 1991; Han *et al*. 2002). Glucocorticoids have been reported to enhance NGF expression in the developing and adult hippocampus (Fabrazzo *et al*. 1991; Barbany and Persson 1992; Saporito *et al*. 1994; Shi *et al*. 1998), while adrenalectomy had the opposite effect (Aloe 1989; Sun *et al*. 1993). Moreover, glucocorticoids increase the expression of neurotrophin receptors in the MS (Shi *et al*. 1998; Roskoden *et al*. 2004). Thus, it seems likely that the effect of GR signaling described in the present study is due to impaired expression of NGF and its receptors in GR^{NesCre} mice. However, it is obvious that the most dramatic effect on the total number of MS cholinergic neurons is observed at the endpoint of development and in adult mutants. Since this is after the developmental peak of NGF expression (Large *et al*. 1986), GR signaling is either necessary to maintain low, adult NGF signaling or acts via another pathway to ensure maintenance of cholinergic MS neurons.

GR signaling and cholinergic functions in the hippocampus

Although there was a significant reduction of medial septal ChAT positive neurons in GR^{NesCre} mice, several observations of the present study suggest, that this loss of cholinergic cells in the cell body region could be compensated for to maintain cholinergic function at a normal level: (i) both the density and the morphology of cholinergic fibers stained for AChE appeared unchanged in hippocampal tissue; (ii) the activity of the cholinergic marker enzyme ChAT was at the same level in hippocampal homogenates of control and GR^{NesCre} mice, respectively; (iii) although tissue accumulation of [^{3}H]choline into hippocampal slices is not a *specific* marker for the density of cholinergic axon terminals, there was no difference between the two groups of mice; (iv) the amount of basal and evoked release of ACh was identical in hippocampal slices from both groups; (v) the indirect muscarine receptor agonist physostigmine inhibited the evoked release of ACh in hippocampal slices from control and GR^{NesCre} mice to exactly the same extent, suggesting that physostigmine – by its inhibition of AChE – increased the endogenous concentration of ACh to the same level in the vicinity of the muscarinic M_2 auto receptor; (vi) the latter remark is further supported by the fact that the presence of an antagonist at presynaptic M receptors, atropine, led to an identical increase of ACh release in hippocampal slices of both mice strains.

The cholinergic septo-hippocampal projection has been shown to sprout within the hippocampus after entorhinal lesions (Frotscher *et al*. 1996; Naumann *et al*. 1997). Moreover, loss of cholinergic septo-hippocampal innervation cannot be compensated for by cholinergic interneurons in the hippocampus itself (Frotscher 1988). Based on these observations we suggest that the loss of cholinergic cell bodies in the medial septal region of GR^{NesCre} mice leads to a compensatory sprouting of the remaining fibers to the hippocampal formation such that the cholinergic transmission in this brain region is maintained at the level of controls.

Conclusions

Taken together we show in the present study that GR signaling in the presence of endogenous, basal levels of glucocorticoids is necessary for the proper development and for the survival of cholinergic MS neurons. The developmental cholinergic cell loss in the MS of GR^{NesCre} mutants can be compensated for at the morphological and functional level, most probably by sprouting of the remaining cholinergic MS neurons.

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