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Long-lasting effects of neonatal dexamethasone treatment on spatial learning and hippocampal synaptic plasticity. Involvement of the NMDA receptor complex

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ABSTRACT

The effects of neonatal dexamethasone (DEX) treatment on spatial learning and hippocampal synaptic plasticity were investigated in adult rats. Spatial learning in reference and working memory versions of the Morris maze was impaired in DEX-treated rats. In hippocampal slices of DEX rats, long-term depression was facilitated and potentiation was impaired. Paired-pulse facilitation was normal, suggesting a postsynaptic defect as cause of the learning and plasticity deficits. Western blot analysis of hippocampal postsynaptic densities (PSD) revealed a reduction in NR2B subunit protein, whereas the abundance of the other major *N*-methyl-D-aspartate (NMDA) receptor subunits (NR1, NR2A), AMPA receptor subunits (GluR2/3), scaffolding proteins, and Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) were unaltered. This selective reduction in NR2B likely resulted from altered receptor assembly rather than subunit expression, because the abundance of NR2B in the homogenate and crude synaptosomal fractions was unaltered. In addition, the activity of αCaMKII, an NMDA receptor complex associated protein kinase, was increased in PSD of DEX rats. The results indicate that neonatal treatment with DEX causes alterations in composition and function of the hippocampal NMDA receptor complex that persist into adulthood. These alterations likely explain the deficits in hippocampal synaptic plasticity and spatial learning induced by neonatal DEX treatment.

Key words: glucocorticoid • rat • LTP • αCaMKII • NR2B

Synthetic glucocorticoids (GC), like dexamethasone (DEX), are widely used for treatment and prevention of chronic lung disease in prematurely born human infants (1). Typically, high doses of GC are administered for several weeks, notably during a period of life that is critical for the development of the brain of the infant. Recently, major concern has arisen about

the safety of this therapy on the long-term development of the child (2–4). Although long-term follow-up studies are not available, recent evidence indicates increased neuromotor dysfunction at 2 years of age, and higher rates of sensorineural disabilities and lower intelligence quotient at 5 years of age in infants born prematurely and treated with DEX (5, 6). These data indeed suggest adverse neurodevelopmental effects of postnatal treatment with GC.

In rats, neonatal GC treatment results in impaired performance in spatial learning tasks later in life (7, 8). Given the crucial involvement of the hippocampus in spatial learning and memory (9) and the vulnerability of this brain structure for perinatal insults (10, 11), this suggests that neonatal treatment with GC may influence hippocampal development and have long-lasting adverse effects on hippocampal function.

It is generally thought that processes underlying bidirectional modification of the strength of hippocampal glutamatergic synapses, referred to as long-term potentiation (LTP) and long-term depression (LTD), are critical for spatial learning and memory (12, 13). These activity-dependent synaptic plasticity processes, and spatial learning as well, are known to require activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors (12). NMDA receptors are glutamate-gated ion channels, coassembled of members of three receptor subunit families that are differentially expressed in the brain: NR1, NR2 (NR2A-D), and NR3A (14, 15). They are dynamic structures and highly enriched in the postsynaptic density (PSD) fraction, which also contains scaffolding proteins and different classes of kinases such as the nonreceptor tyrosine kinase src and the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII). The latter comprises up to 30% of total PSD protein (16) and is thought to play an essential role in hippocampal synaptic plasticity and spatial learning and memory (17–19).

In the present study, we investigated the long-term effects of neonatal DEX treatment on hippocampal functions, using a treatment protocol with tapering doses of DEX proportional to the one used to treat preterm human neonates (20). Notably, the developmental stage of the rat brain around birth is comparable with that of the preterm human infant, born during weeks 26–32 of gestation (21). The following questions were addressed. Does neonatal DEX treatment lead to impaired hippocampus-dependent spatial learning later in life? If yes, is this impairment associated with a deficit in hippocampal synaptic plasticity and does neonatal DEX affect the postsynaptic hippocampal NMDA receptor complex? Answers to these questions can add to our insight into the neurodevelopmental effects of GC and the potential long-term risks of neonatal DEX treatment of human preterm infants.

MATERIALS AND METHODS

Animals

Ten-day pregnant Wistar rats (250–280 g, Central Animal Laboratory, Utrecht University, The Netherlands) were housed individually. Pups were born on days 22–23 of gestation. On the day of birth (day 0), they were removed from their nests and eight pups (4 females and 4 males) were randomly placed back with each dam. Pups were weaned at 21 days of age and remained group housed with littermates until experimentation at 3–4 months of age. Rats had ad libitum access to food and water. Light-dark cycle (dark phase 1900–0700), temperature (21°C), and humidity

(60%) were kept constant. All experimental procedures were approved by the Committee for Animal Experimentation of the University of Utrecht.

Experimental design

Newborn rats were intraperitoneally injected with dexamethasone-21-phosphate (DEX; Organon B.V., Oss, The Netherlands) on neonatal day 1 (0.5 μg/g body wt), day 2 (0.3 μg/g), and day 3 (0.1 μg/g) or with equal volumes (10 μl/g) of sterile pyrogen free saline (SAL). All pups of a nest received the same treatment, i.e., either DEX or SAL. This experimental design excluded growth retardation due to competition between DEX and SAL pups. There were no differences in maternal pup-directed behavior between DEX- and SAL-treated nests (P. J. G. H. Kamphuis, J. M. Bakker, G. Croiset, F. van Bel, and V. M. Wiegant, unpublished observations). Behavioral, electrophysiological, and biochemical studies were performed using different groups of adult male rats (3–4 months) exposed to DEX or SAL during neonatal life. Experimental groups were composed of rats from different nests, and every nest contributed only one rat to a group.

Spatial learning in the Morris water maze

Spatial learning was tested in the Morris water maze (210 cm diameter) with a hidden (i.e., reference memory task) or a visible platform (i.e., visual cued task) as described previously (22). Briefly, on 5 consecutive days, the animals were given three acquisition trials each day in which the rat was given a maximum of 120 s to find the submerged platform and was allowed to stay on the platform for 30 s. Latency to reach the platform and distance swum were determined. Performance in the three trials was averaged to yield one data-point per rat per day. Three days after the fifth day of the training a "transfer trial" was performed. The platform was removed, and the rat was put into the maze and allowed to swim for 60 s. A zone with a radius of 20 cm was defined around the former position of the platform center, and both the time spent and distance swum in this zone were calculated. Different groups of rats were tested in the water maze with a visible platform (which does not require spatial orientation; (23) to reveal possible deficits in sensorimotor processes. Rats were given three trials per day on 3 consecutive days and allowed to search for a maximum of 60 s per trial. Performance in the three trials was averaged to yield one data-point per rat per day.

Spatial working memory (24) was tested in a single-trial spatial task under the same conditions as the other tasks, except that the water maze consisted of a smaller circular black pool (140 cm diameter, 30 cm height, filled with water to a depth of 20 cm). Before testing, rats were familiarized with the water maze during five trials on 3 consecutive days. Rats were then submitted to one acquisition and one retention trial per day on 6 consecutive training days with a different location of the platform each day. In the acquisition trial, the rat was given a maximum of 90 s to find the submerged platform and allowed to stay on the platform for 30 s before being placed back in its home cage. After a delay of 5 (days 1, 3, and 5) or 20 (days 2, 4, and 6) min, the rat was subjected to the retention trial. Data obtained with the same inter-trial interval (5 or 20 min) on the 3 test days were averaged per rat yielding single data points for performance during the acquisition and retention trial.

All behavioral testing was done in the dark period between 20 and 24 h, and both latency and distance swum to reach the platform were automatically measured using a computer video-tracking system (Ethovision, Noldus, Wageningen, The Netherlands).

Electrophysiological recording

Transverse hippocampal slices (450 μm thick) were prepared as described elsewhere (22), maintained submerged in a recording chamber, and perfused (1–2 ml/min) with artificial cerebrospinal fluid of the following composition (mM): 124 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 10 glucose, 20 $NaHCO_3$, and 2.5 $CaCl_2$ and gassed with 95% O_2 and CO_2. All experiments were done at 30°C and after allowing the slices to recover for at least 60 min after preparation. Glass microelectrodes with a tip diameter of 2–3 μm and resistance of 3–6 MΩ, filled with perfusion medium, were used to record field potentials. Field excitatory postsynaptic potentials (fEPSPs) were recorded using a conventional A-C coupled amplifier (in-house made). fEPSPs were digitized and stored in a computer using SIGAVG and Spike2 software (CED, Cambridge, UK). Monophasic constant current pulses of 150 ms duration and 20–200 mA intensity were administered through a stimulation unit (Master 8 pulse generator, Jerusalem, Israel).

Synaptic plasticity

LTP and LTD were defined as changes in the slope of fEPSPs after the application of conditioning stimulation (12, 13). Bipolar stainless-steel stimulation electrodes, insulated except for the tip (Clark, Electro medical instruments, Pangbour, UK), were placed on the afferent fibers of the stratum radiatum of the CA1 region of the hippocampus. Only those slices in which the amplitude of the fEPSP was 1 mV or more at maximum response were included in the experiment. Before each experiment, the stimulus intensity to elicit threshold and maximum fEPSPs was determined. A stimulus-response relation was determined, and the stimulus intensity was adjusted to evoke fEPSPs of half-maximum amplitude and kept constant thereafter. Stimulation frequency was 0.05 Hz. Baseline response value was obtained from the first 15 min of every recording. The average slope of the fEPSP at baseline was set at 100%, and changes in slope were expressed as a change from baseline. To plot the frequency-response function of the hippocampal synapses, 900 pulses were given at different frequencies (0.05-100 Hz) to the afferent fibers of the CA1 field. To avoid conditioning by prior stimulation, we used different slices for each stimulation frequency. After the application of the conditioning stimuli, the responses were recorded for 30 min when a stable response was obtained. High-frequency stimulation (HFS; 100 Hz for 1 s and 50 Hz for 2 s) was applied with intervals of 10 s. Low-frequency stimulation (LFS) was given as a single train of 900 stimuli at 10 Hz (for 1.5 min), 5 Hz (for 3 min), or 1 Hz (for 15 min).

Paired-pulse facilitation

Paired-pulse facilitation (PPF) in the CA1 field of the hippocampus was evaluated in separate slices under identical conditions as used for LTP (see above). PPF was measured as an increase in response to the second stimulus of two identical stimuli (25), and in this study we used inter-stimulus intervals between the paired pulses of 50 and 100 ms. PPF was expressed as the ratio of the slope of the second and the first fEPSP.

PSD preparation, endogenous phosphorylation, and immunoprecipitation studies

To isolate PSD from rat hippocampus the method of Carlin et al. (26) was used, modified as described previously (27). Quantitation of Western blots was performed by means of computer-assisted imaging (Quantity-One System; Bio-Rad, Hercules, CA).

CaMKII-dependent endogenous phosphorylation of PSD proteins and immunoprecipitation assays were performed as described previously (28). Briefly, 5 μg of hippocampal PSD proteins were incubated in a buffer solution containing: 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10^{-5} M okadaic acid (Sigma, St. Louis, MO), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM DTT with 0.1 mM of [γ–^{32}P]ATP (2 μCi/tube; 5000 Ci/mmol; APBiotech, Uppsala, Sweden) in the presence of either 1 mM $CaCl_2$ and 40 μg/ml calmodulin, or 1 mM EGTA. Reactions were performed at 37°C for 5 min and were stopped by immersion in liquid nitrogen.

For tyrosine-dependent phosphorylation, assays were performed as described previously (28). Briefly, 5 μg of purified hippocampal PSD were incubated in 20 mM HEPES, pH 7.4, containing either 10 mM $MnCl_2$ or 1 mM EGTA-10 mM EDTA in the presence of 0.2 mM Na_3VO_4, 0.1 mM PMSF, and 20 mM DTT with 0.1 mM γ^{32}P-ATP (1 μCi/tube; 5000 Ci/mmol; APBiotech). Reactions were performed at 37°C for 15 min and were stopped by the addition of electrophoresis sample buffer [2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8].

For immunoprecipitation, phosphorylated PSD proteins were incubated in buffer A containing the following: 0.2 M NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% NP-40, and 1% SDS in a final volume of 400 μl with antibodies against NR2A (dilution 1:100) overnight at 4°C. Protein A-agarose beads (5 mg/tube), washed in the same buffer, were added and incubation was continued for 2 h. The beads were collected by centrifugation and washed three times with buffer A. Sample buffer for SDS-PAGE was added, and the mixture was boiled for 3 min. Beads were pelleted by centrifugation, and a volume of supernatants was applied to SDS-PAGE 6%.

αCaMKII assay

αCaMKII activity in hippocampal PSD was performed by means of SignaTECT αCaMKII assay system (Promega, Madison, WI).

Antibodies

Polyclonal antibody against active p286-αCaMKII was purchased from Promega; polyclonal antibodies against GluR2/3, NR2B, NR2A, and NR1 and monoclonal antibody against αCaMKII from Chemicon International Inc. (Temecula, CA); polyclonal antibody against actin from Sigma-Aldrich; monoclonal antibody against PSD-95 from Affinity BioReagents Inc.; and antibody against phoshotyrosine (PY20/4G10) from Upstate Biotechnology (Lake Placid, NY).

Statistical analysis

Data are means ± SE. Behavioral performance during water maze training was analyzed using an ANOVA for repeated measurements, with one-between subjects factor (group) and one-within

subjects factor (day), followed by *t* test with Bonferroni's correction as post hoc comparison test in case of a significant main effect. Differences in the single-trial place learning were analyzed using a two-factor ANOVA for repeated measurements, with two between-subject factors (group and interval length) and one-within factor (trial). This analysis was followed by ANOVA when appropriate. For each measure of plasticity, the effects of PPF, LFS, or HFS were analyzed within each group by comparing the absolute values of the slope of the fEPSP before and after stimulation with a Wilcoxon matched-pair signed test. Western blot data were analyzed using ANOVA. All other behavioral, electrophysiological, and biochemical group differences were analyzed by ANOVA. *P* values of <0.05 were considered significant.

RESULTS

Spatial learning is impaired in adult rats after neonatal DEX treatment

No differences were found in body weight between adult rats (3–4 months of age) exposed to DEX or SAL during neonatal life (data not shown). In the first set of experiments, DEX or SAL rats were tested in the hidden platform version of the Morris water maze. As is shown in Fig. 1A and B, escape latency and distance swum to reach the hidden platform decreased over the 5 days of training in both DEX and SAL rats (day: $P<0.001$ for escape latency and distance swum). Performance during the first training trial did not differ between the two treatment groups (data not shown), but, overall, DEX rats showed increased escape latency compared with SAL rats (group: $P<0.05$). Post hoc analysis indicated that this difference was significant at day 3. Although distance swum to reach the hidden platform appeared to be similarly increased in DEX compared with SAL rats, this difference did not reach statistical significance. The average swimming speed during the training trials was not different between DEX (17.3±0.9 cm/s) and SAL rats (18.8±0.9 cm/s). On the fifth training day, the treatment groups showed similar performance. Three days later, a transfer trial was conducted: the platform was removed and rats were given a free-swim trial for 60 s. Performance during this task was not different between DEX and SAL rats (time and distance swum in the defined zone around the former position of the platform; data not shown).

It is known that neonatal DEX treatment can have lasting effects on sensorimotor processes (29). To exclude whether the increased latency of DEX rats in the Morris-maze was due to motivational factors or sensorimotor deficits, groups of naïve DEX and SAL rats were trained in the water maze with a visible platform. As is shown in Fig. 1C and D, escape latency and distance swum to reach the visible platform declined during the 3 training days (day: $P<0.001$ for escape latency and distance swum). The performance in this task was not different between DEX and SAL rats.

The standard protocol of the Morris water maze uses a fixed location of the platform across days, assessing mainly spatial reference memory (9). We also performed a single-trial learning task, which is similar to a delayed-matching-to-sample task (24). In this task, DEX and SAL rats were given two trials per day (an acquisition and a retention trial), with varying location of the submerged platform per day. By changing the location of the platform each day, this task requires memory of where the possible escape location is to be updated frequently in an otherwise unchanging environment, suggesting that this task has working memory properties (24, 30). A decline of escape latency and distance swum to reach the platform between trial 1

and 2 reflects single-trial learning. As is shown in Fig. 2, neonatal DEX treatment resulted in an impaired performance of this task in adulthood. There were significant differences in escape latency (group: $P<0.001$; group x trial: $P<0.05$) and distance swum (group: $P<0.05$; group x trial: $P<0.05$) between DEX and SAL rats. Compared with the first trial, both escape latency and distance swum to reach the hidden platform in the second trial dropped significantly in SAL rats irrespective of the inter-trial interval ($P<0.01$). DEX rats did not show a decline in escape latency and distance swum between the acquisition and retention trial. Although there was no difference between DEX and SAL rats in performance during the acquisition trial, escape latency and distance swum during the retention trial were higher in DEX than in SAL rats ($P<0.05$), although the difference in distance swum using a 20 min inter-trial interval did not reach statistical significance. There was no difference in performance of rats between the tests with 5 and those with 20 min inter-trial interval.

LTP is impaired and LTD facilitated in hippocampal slices from adult rats after neonatal DEX treatment

We studied the long-term effects of neonatal DEX on synaptic plasticity. First, we tested PPF in hippocampal slices of adult rats treated with DEX or SAL to reveal possible differences between treatment groups in presynaptic processes involved in neurotransmitter release (25). In both groups, the slope of the fEPSP was significantly higher in response to the second stimulus (see Fig. 3B; $P<0.05$) with both stimulus interval lengths used (50 and 100 ms), indicating PPF expression. As is shown in Fig. 3A, the responses were not different between DEX and SAL rats.

We then tested LTD and LTP in hippocampal slices obtained from DEX and SAL rats. The average baseline fEPSP slope was not different between DEX (0.40±0.02 mV/ms) and SAL rats (0.42±0.02 mV/ms). There was a significantly ($P<0.05$) increased depression of the fEPSP in response to LFS (1, 5, and 10 Hz) in slices from DEX compared with SAL rats (Fig. 3C). A 1 Hz train of 900 stimuli resulted in synaptic depression to 94.1 ± 4.3% of the baseline values in slices from SAL rats and to 66.6 ± 3.7% in DEX rats ($P<0.001$). The slopes of the fEPSP in slices from DEX rats 30 min after 1 and 5 Hz conditioning stimuli were depressed compared with baseline values ($P<0.05$). No significant depression was measured in slices from SAL rats using the same protocol. HFS with 100 Hz resulted in an increase of the fEPSP ($P<0.05$) compared with baseline values both in slices from DEX and SAL rats, but with 50 Hz of stimulation this increase was only found in slices from SAL rats. Potentiation in DEX rats was markedly reduced as compared with SAL rats, both after stimulation with 50 Hz (DEX vs. SAL: 112.5±4.7 vs. 150.7±11.3%; $P<0.05$) and 100 Hz (DEX vs. SAL: 142±6.8 vs. 227.2±14.3%, $P<0.001$).

Reduced presence of the NR2B subunit and increased αCaMKII activity in hippocampal PSD of adult rats after neonatal DEX treatment

Hippocampal slices of DEX rats did not express a deficit in PPF, suggesting that presynaptic function is intact and that a deficit in the postsynaptic machinery underlies the alterations found in synaptic plasticity. To test this, we studied the long-term effects of neonatal DEX on the NMDA receptor complex in purified hippocampal PSD. To evaluate protein levels of glutamate receptors and PSD-associated signaling proteins, such as αCaMKII (31, 32), Western blot analysis was performed. The same protein yield was obtained in PSD purified from DEX and

SAL rats, and the same amount of PSD protein was applied to SDS gel and electroblotted for both treatment groups.

As is shown in Fig. 4A, immunostaining of the GluR2/3 subunits of the AMPA receptor and the NR1 subunit of the NMDA receptor was similar for DEX and SAL rats. In addition, the concentration of proteins known to be highly enriched in the PSD fraction, such as actin, PSD-95 and αCaMKII, was similar for both treatment groups. This suggests that the gross composition of the PSD fraction was not affected by neonatal DEX treatment. However, Western blotting showed a selective and significant reduction in NR2B subunit protein in DEX compared with SAL rats (-48.5±7.6%, $P<0.01$), whereas no difference was found for the NR2A protein level. To investigate whether the decrease in NR2B immunostaining in the PSD of DEX rats was due to a decreased expression or to an altered targeting of this subunit in PSD, Western blotting was performed in three different subcellular compartments: the homogenate, the P2 fraction (corresponding to the crude synaptosomal compartment), and the purified PSD fraction. As shown in Fig. 4B and C, NR2B immunostaining was again found reduced in PSD from DEX rats (-53.2±6.9%; $P<0.01$), but no significant differences were present in the homogenate and the P2 fraction. Together, these findings indicate an alteration in the compartmentalization of the NR2B subunits of the NMDA receptor complex in DEX rats and suggest that DEX treatment had affected the assembly and/or targeting of the NMDA receptor, rather than the expression of the NR2B subunit.

In view of the fact that different classes of signaling proteins, e.g., αCaMKII, are directly associated with the NR2A and 2B subunits of the NMDA receptor, we also studied the long-term effects of neonatal DEX on αCaMKII-dependent phosphorylation of NR2 subunits in the PSD fraction (27, 31–33). Figure 5A shows a representative autoradiograph of hippocampal PSD phosphorylated under conditions known to maximally activate αCaMKII and immunoprecipitated with anti-NR2A polyclonal antibody. An increase of [^{32}P]phosphate incorporation was observed in a 170 kDa band (+112.4±17.9%, right panel) and a 50 kDa protein band (+123.9±12.4%, $P<0.01$) corresponding to NR2A and αCaMKII, respectively, as previously reported (27) and revealed also by Western blot analysis performed on the immunocomplex (Fig. 5A, left lanes). The observed net increase in the ^{32}P-incorporation into NR2A subunit in DEX rats can be due either to an increase in the activity of the kinase or to a reduced in vivo phosphorylation preceding the post hoc assay. To clarify this issue, αCaMKII activity was tested using two different experimental approaches (Fig. 5B and C). The basal autophosphorylation state of the kinase in hippocampal PSD was analyzed by means of anti-active-p286 antibody. As shown in Fig. 5B, p286-αCaMKII immunostaining was significantly increased in DEX as compared with SAL rats (+88.3±1.6%, $P<0.01$), but there was no difference in αCaMKII immunostaining. This was confirmed by the finding that αCaMKII activity on an added exogenous substrate (syntide-2) was increased in DEX rats (Fig. 5C, +103.2±14.6%, $P<0.01$). Notably, the increase in αCaMKII activity shown in Fig. 5B and C was quantitatively similar to that observed in the post hoc phosphorylation assay for the endogenous substrate NR2A (see Fig. 5A). Together, these data suggest that the increase in αCaMKII-dependent phosphorylation observed in the hippocampus of DEX rats is indeed related to an increased activity of the PSD-associated αCaMKII.

Since it is known that both NR2A and NR2B subunits can be also tyrosine phosphorylated in the PSD fraction (34, 35), we evaluated whether tyrosine phosphorylation could have contributed to the altered phosphorylation of the NR2A subunit in DEX rats. Western blot analysis with phosphotyrosine antibodies (PY20/4G10) was performed on PSD reacted under conditions known to obtain maximal tyrosine-dependent phosphorylation (28). No differences were observed between the experimental groups, indicating that tyrosine phosphorylation was not affected by neonatal DEX treatment (Fig. 6A). In particular, a 170 kDa band (arrowhead) corresponding to NR2 subunits was evident (28, 34), but not affected by neonatal DEX treatment. In line with these results, we observed no differences in the immunostaining of src tyrosine kinase in PSD of DEX and SAL rats (Fig. 6B).

DISCUSSION

In this study, we found that spatial learning is impaired in adult rats that had been treated with DEX as neonates. This cognitive deficit appeared to be associated with an impaired LTP and a facilitated LTD in the hippocampal CA1 field. In addition, the concentration of NR2B subunit protein was selectively reduced and the activity of αCaMKII increased in hippocampal PSD of DEX rats. These results are taken to indicate that exposure to DEX during brain development has long-lasting effects on composition and function of the NMDA receptor complex, thereby compromising hippocampal plasticity and cognitive performance in later life.

DEX rats showed impaired performance in both the multiple and the single trial hidden platform versions of the Morris maze that address reference and working memory, respectively (24, 30). Since performance in the visible platform version was normal, indicating that sensorimotor and motivational functions were not altered, this clearly points to a cognitive deficit in DEX rats. These findings accord with and extend those of others who reported similar acquisition deficits in the reference memory version of the water maze in adult rats that had been treated with DEX later in the first week of life (7, 8).

Spatial learning in the two tasks used involves cognitive processes in which the hippocampus plays a crucial role (9, 30, 36). LTP in the hippocampal CA1 field was impaired, and LTD was facilitated in hippocampal slices of DEX rats. It is generally thought that LTP and LTD contribute bidirectionally to synaptic reinforcement and memory storage and that a systematic downward shift in the frequency response, favoring LTD over LTP, causes learning impairment (12, 13, 37). In line with this notion, the observed impairment in hippocampal plasticity can explain the deficit in spatial learning in DEX rats.

PPF was similar in hippocampal slices of DEX and SAL rats, and such data are generally taken to indicate that presynaptic function is not affected (25). Therefore, the alterations found in synaptic plasticity and spatial learning are most likely caused by a defect in the postsynaptic machinery. Synaptic plasticity in the hippocampal CA1 field is known to be largely dependent on NMDA receptors (12, 13, 37), and spatial learning in both tasks used in the present study also requires activation of NMDA receptors (30, 38). We found a reduced concentration of the NR2B subunit of the NMDA receptor in hippocampal PSD of DEX rats, whereas the levels of NR1 and NR2A as well as AMPA receptor subunits and scaffolding proteins were unaltered. This indicates that DEX treatment had not altered the gross structure of PSD but selectively affected the abundance of NR2B subunit protein. NMDA receptors are heteromeric assemblies of a core

NR1 subunit and various modulatory NR2 subunits (14, 15). Thus DEX treatment had altered the subunit composition of hippocampal NMDA receptors.

Subunit composition is a critical determinant of the functional properties of the NMDA receptor (39, 40). In the adult hippocampus, NR2A and NR2B are the most abundant NR2 subunits (41). It has been shown that recombinant mouse NR1-NR2A receptors expressed in *Xenopus* oocytes gate smaller Ca^{2+} currents, posses lower affinity for glutamate, and desensitize faster than NR1-NR2B receptors (42, 43). A selective increase in the ratio NR2A/NR2B, as we observed in DEX vs. SAL rats, would therefore result in a tighter regulation of the Ca^{2+} influx, thereby reducing the chances for induction of LTP and increasing those for LTD (40, 41, 43, 44). Recent reports have provided evidence that supports the above line of reasoning. It has been demonstrated that NR2B overexpression in the forebrain of transgenic mice leads to facilitation of LTP and enhanced learning capability (45). Conversely, selective knockdown of NR2B subunit expression by injection of antisense oligonucleotides into the hippocampus of adult rats proved to be sufficient to abolish LTP in the hippocampal CA1 region and impair spatial learning (46). Thus the reduced presence of NR2B subunit detected in the hippocampal NMDA receptor likely is a critical factor in the mechanism underlying the synaptic plasticity and learning deficits found in DEX rats.

There is evidence that neurotransmission via NMDA receptors in the hippocampus is required for acquisition and not for retrieval of information in spatial learning tasks (30). DEX and SAL rats showed similar performance in the free-swim trial given 3 days after the last acquisition session of the multiple trial task in the Morris maze, indicating normal retrieval of information. Taken together, this strengthens the conclusion that the defect found in the NMDA receptor underlies the cognitive deficit of DEX rats.

Interestingly, the depletion of NR2B was restricted to the PSD fraction and not found in the hippocampal homogenate and crude synaptosomal fractions of DEX rats. This suggests that the neonatal DEX treatment had not affected NR2B protein expression but rather the correct targeting of the subunit and the assembly of NMDA receptors. Very recent observations have demonstrated the existence of a substantial intracellular pool from which NMDA receptor subunits are recruited to the synaptic membrane after LTP stimulation, suggesting that synaptic insertion of NMDA receptors may be a key step in the regulation of synaptic plasticity (47). In addition, it is now known that NR2A- and NR2B-subunit containing receptors are delivered to the synaptic membrane following different rules. Notably, synaptic insertion of NR2B-containing receptors is not promoted by increased NR2B expression, while increased expression of NR2A subunit promotes insertion of NR2A-containing receptors (48). These data, together with our present results, suggest that trafficking rather than expression of NR2B represents a key element in the regulation of the composition and function of the NMDA receptor complex at postsynaptic sites. Our present data accord with the idea that formation of the NMDA receptor complex is a physiologically regulated process that can be target of pathological insults (40). Comparable results have been reported recently in a well-established rat model for Parkinson's disease, the 6-hydroxydopamine lesioned rat, where selective redistribution of NR1 and NR2B subunits from the striatal membrane to the cytoplasmic compartment was linked to a reduction in NMDA receptors composed of NR1 and NR2B subunits (49).

The mechanism underlying the effect of neonatal treatment with DEX on the compartmentalization of the NR2B subunit later in life is unclear. In adult rats, chronic alterations in circulating corticosterone levels can affect NR subunit expression and thereby lead to changes in NMDA receptor subunit composition (50, 51). However, we have found no differences in the circulating concentrations of corticosterone throughout the diurnal cycle between adult DEX and SAL rats (52)**.** Recently, McGowan et al. (53) showed that treatment with DEX during brain maturation could modify the NMDA receptor. They found a decreased binding capacity and affinity of the receptor for the NMDA ligand MK-801 12–18 h after DEX treatment in 5-day-old lambs. In this respect, it is of interest that the sensitivity of NMDA receptors for glutamate depends on their subunit composition, with reduced presence of NR2B in the receptor leading to reduced sensitivity (41, 44). Therefore, we conclude that the depletion of the NR2B subunit found in adult DEX rats is most likely caused by a neurodevelopmental change induced by DEX in the neonatal brain.

αCaMKII is closely associated with the NMDA receptor in the PSD, and its activity is another essential component of the molecular machinery of hippocampal synaptic plasticity (17–19). Phosphorylation of the NR2A subunit, an endogenous substrate of αCaMKII, and of syntide, an exogenous substrate of the enzyme, and of αCaMKII present in PSD was increased in DEX rats, pointing toward enhanced activity of PSD-associated αCaMKII. In addition, the immunostaining for p-286-αCaMKII was also increased, indicating an enhanced state of autophosphorylation of αCaMKII in PSD. Autophosphorylation indeed results in increased, autonomous activity of the enzyme (17, 54). Together, the data therefore suggest that, in PSD of DEX rats, αCaMKII is locked in the activated, Ca^{2+}-independent state. Altered αCaMKII activity in PSD can disturb the delicate, dynamic balance between kinase and phosphatase activity that is thought to govern synaptic plasticity (17, 19). Indeed it has been shown that transgenic mice overexpressing a mutated form of αCaMKII with autonomous activity display a downward shift of the frequency response curve, favoring LTD over LTP in the hippocampal CA1 field at low frequencies of stimulation, as well as spatial learning disabilities (18).

The present effects on the developmental programming of hippocampal neurons may be triggered by interaction of DEX with intracellular GC receptors that regulate transcriptional activity or reflect nongenomic actions of DEX. Indeed, intracellular GC receptors are highly enriched in hippocampal neurons. GC action in adult rats has been associated with an increase in extracellular excitatory amino acids including glutamate (55, 56) and increased NMDA receptor mediated Ca^{2+} influx (50, 57), resulting in disturbed intracellular Ca^{2+} homeostasis and irreversible neuronal damage (58, 59). In neonatal life, when endogenous GC are regulated at extremely low levels (10, 60), hippocampal neurons may be particularly vulnerable to the effects of GC, because NR2B is the predominant NR2 subunit of the hippocampal NMDA receptor during that developmental period (41), and NMDA receptors containing NR2B show enhanced Ca^{2+} gating (42, 44). Although the exact mechanism involved remains to be elucidated, our results highlight the posttranslational machinery that regulates assembly, trafficking, and function of the NMDA receptor as a potential target for regulatory and programming effects of GC hormones.

In preterm human neonates, the acute and short-term beneficial effects of treatment with GC on the injured lung are well established (61). Yet, our present observations reveal that, in the rat,

neonatal treatment with GC can alter the molecular makeup of the brain and lead to a lasting cognitive deficit. They strengthen recent concerns about the long-term safety of this treatment (2–4), call for utmost prudence in treating prematurely born human infants with GC, and urgently warrant investigation of lasting and potentially adverse effects of the treatment.

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Fig. 1

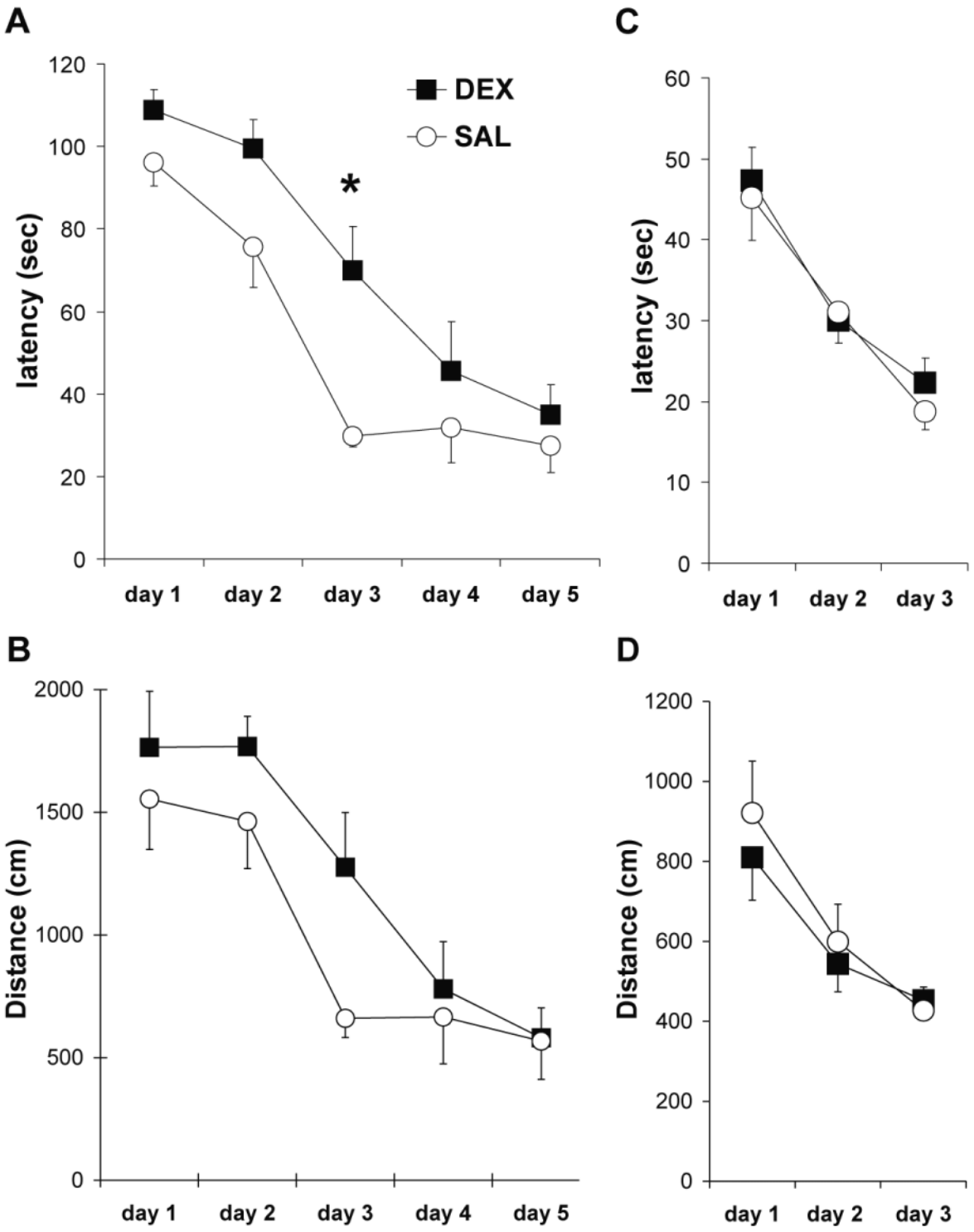

Figure 1. Neonatal DEX treatment results in impaired performance in the hidden but not the visible platform version of the Morris water maze in adulthood. Escape latency (**A**) and distance swum to reach the hidden platform (**B**) during 5 consecutive days of training in adult rats neonatally treated with DEX (-■-; *n*=9) or SAL (-0-; *n*=8). Escape latency was increased ($P<0.05$) in DEX compared with SAL rats. Post hoc comparison revealed a difference between DEX and SAL rats on day 3 (*$P<0.05$). Escape latency (**C**) and distance swum to reach the visible platform (**D**) during 3 consecutive days of training in DEX (-■-; *n*=8) and SAL rats (-0-; *n*=8). Data are means ± SE.

Fig. 2

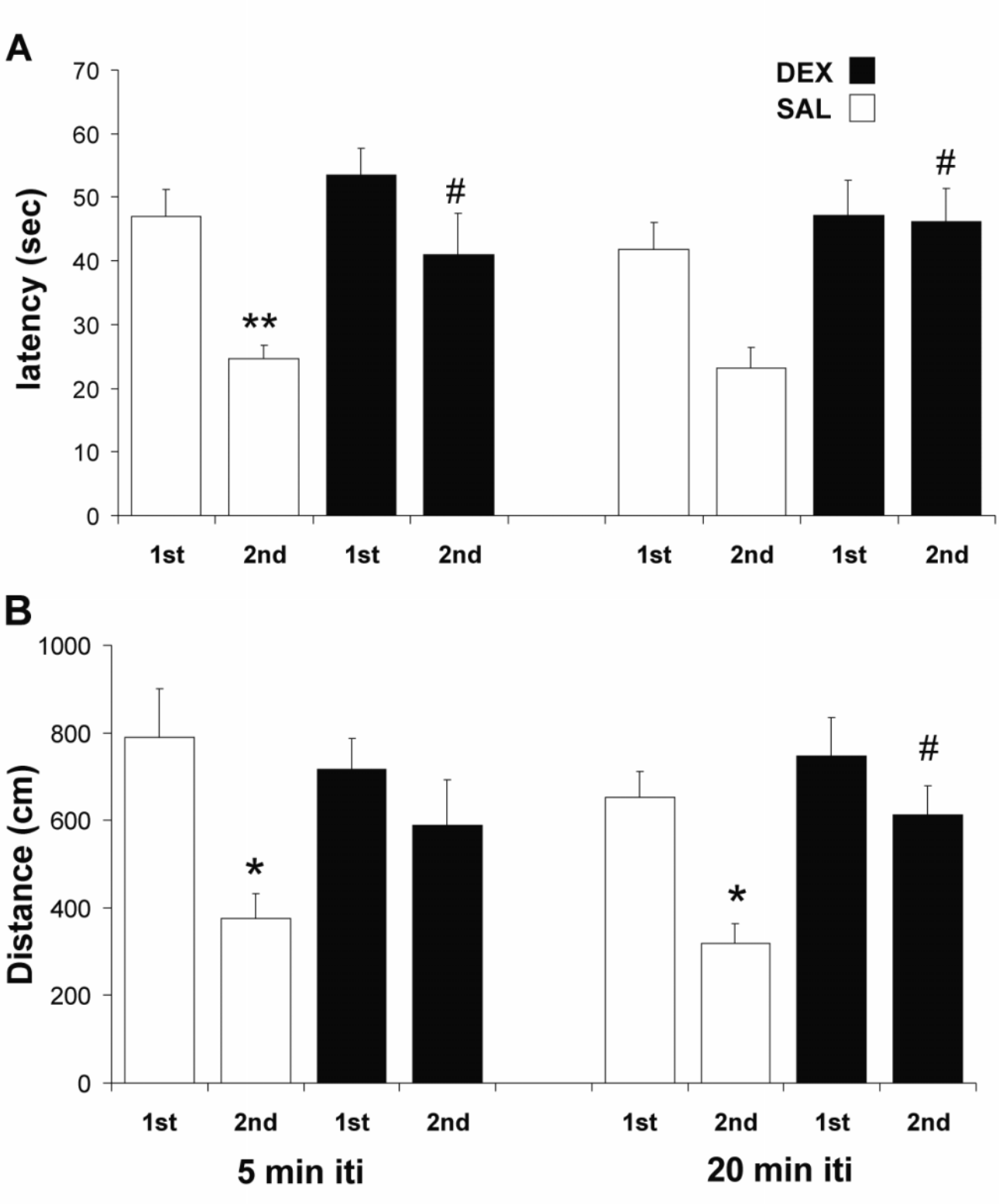

Figure 2. Neonatal DEX treatment results in impaired performance in a single-trial spatial learning version of the Morris water maze in adulthood. Escape latency (**A**) and distance swum to reach the hidden platform (**B**) in adult rats neonatally treated with DEX (dark bars; *n*=11) or SAL (open bars; *n*=12). Rats were given one acquisition (1st) and retention (2nd) trial per day, with an inter-trial interval (iti) of 5 or 20 min, and varying location of the platform per day. Data of test days were averaged per rat and are means ± SE. Differences between the first and second trial (**P*<0.05; ***P*<0.01), and between DEX and SAL rats (#*P*<0.05) are indicated.

Fig. 3

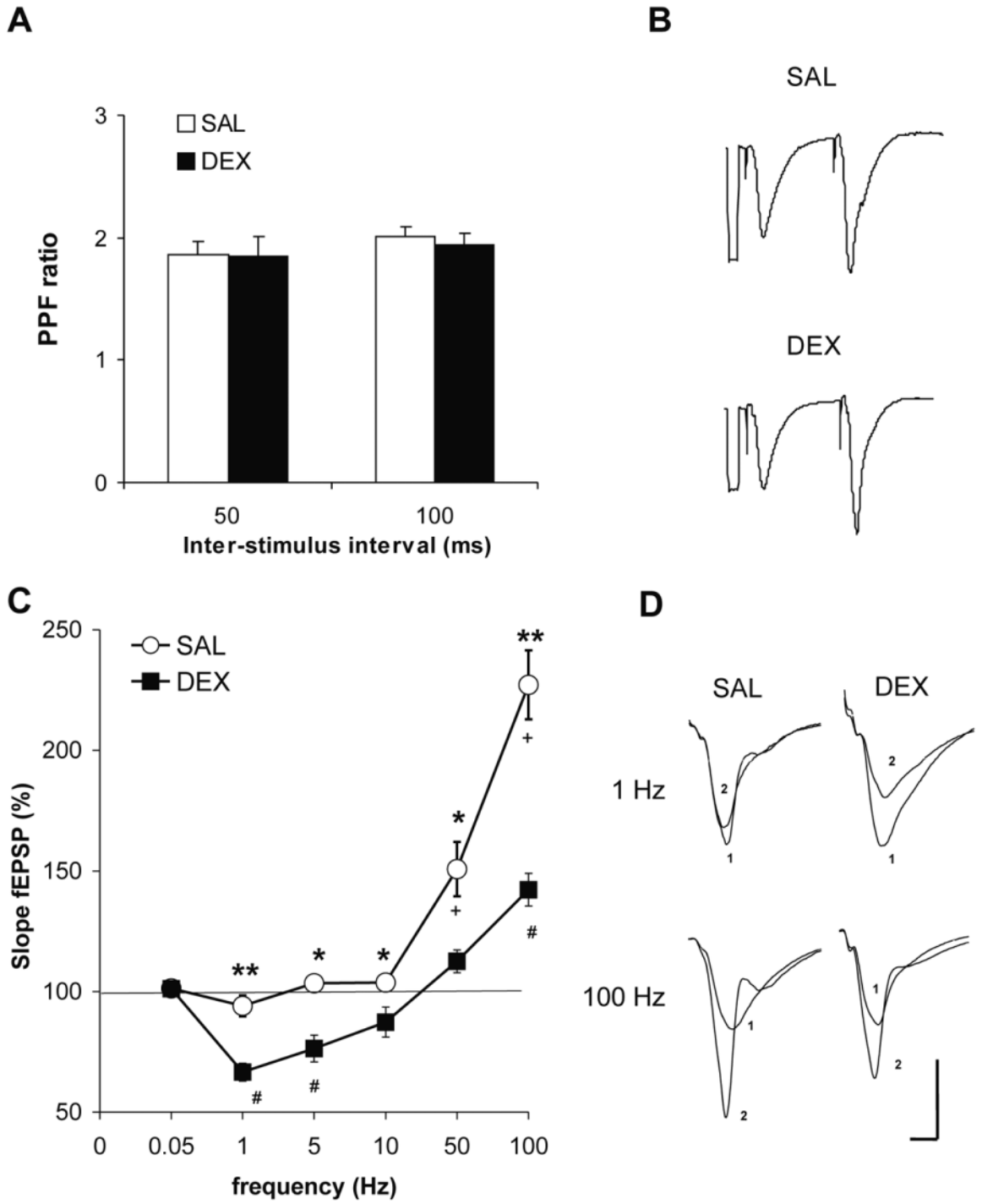

Figure 3. Neonatal DEX treatment results in facilitation of LTD and impairment of LTP induction in the CA1 field of hippocampal slices in adulthood. **A)** Paired-pulse facilitation (PPF) was determined at inter-stimulus intervals of 50 and 100 ms and expressed as the ratio of the slope of the second and the first fEPSP. PPF was found with both inter-stimulus intervals ($P<0.05$) and was not different between DEX (dark bars; $n=6$) and SAL rats (open bars; $n=6$). **B)** Example of PPF obtained with an inter-stimulus interval of 50 ms in slices of DEX and SAL rats. **C)** Relative fEPSP slopes (% of baseline) 30 min after different conditioning stimuli (1–100 Hz) in slices from DEX (-■-; $n=6$) or SAL rats (-0-; $n=6$). Symbols indicate significant differences vs. baseline (#$P<0.05$ within the DEX group; +$P<0.05$ within the SAL group), and differences in response between DEX and SAL treatment groups (*$P<0.05$; **$P<0.01$). **D)** Traces represent averaged fEPSPs ($n=6$) recorded before (1) and 30 min after (2) the application of a 1 Hz or 100 Hz conditioning stimulus. Horizontal scale bar represents 5 ms, vertical bar 1 mV.

Fig. 4

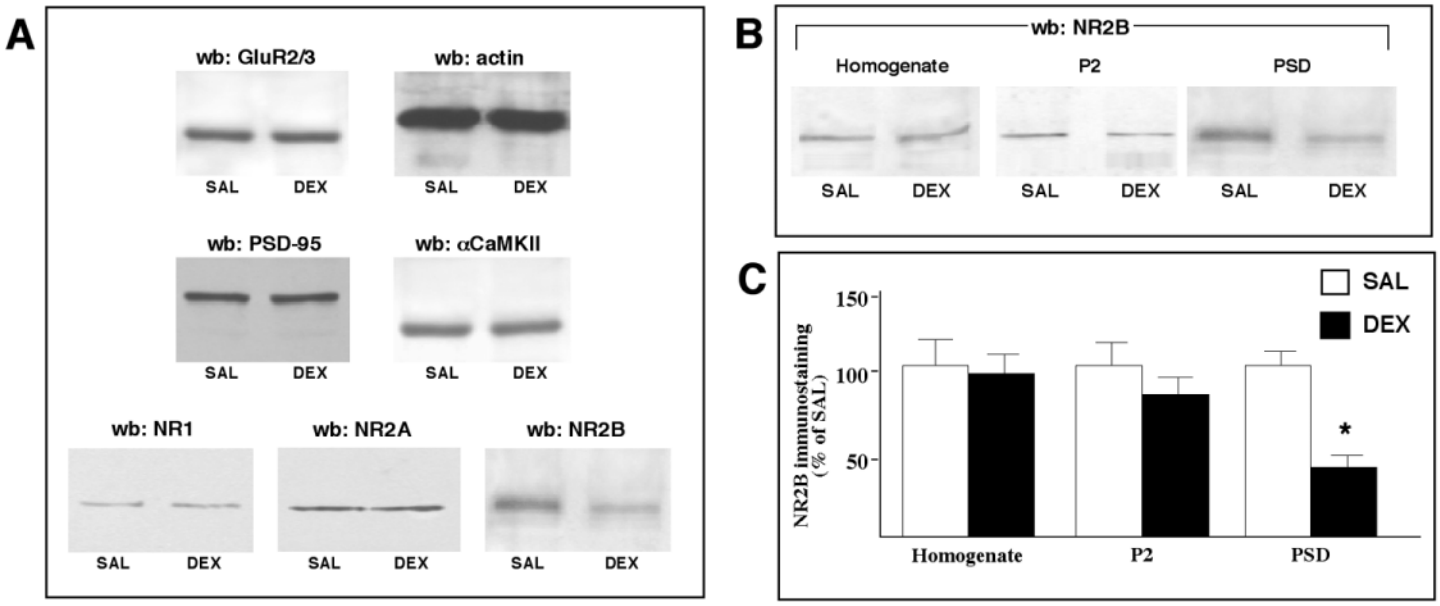

Figure 4. Immunostaining of the NMDA receptor NR2B subunit is selectively reduced in hippocampal PSD from adult rats neonatally treated with DEX. A) Western blot analysis of GluR2/3, actin, PSD-95, αCaMKII, NR1, NR2A, and NR2B in PSD of SAL and DEX rats. NR2B is selectively decreased in DEX rats (-48.5%; $P<0.01$). **B)** Representative Western blot analysis of NR2B in the hippocampal homogenate, crude synaptosomal (P2) and PSD fractions from SAL and DEX rats. **C)** NR2B immunostaining in hippocampal fractions of DEX rats expressed as % of SAL rats. Data are means ± SE (n=8 for each group; *$P<0.01$).

Fig. 5

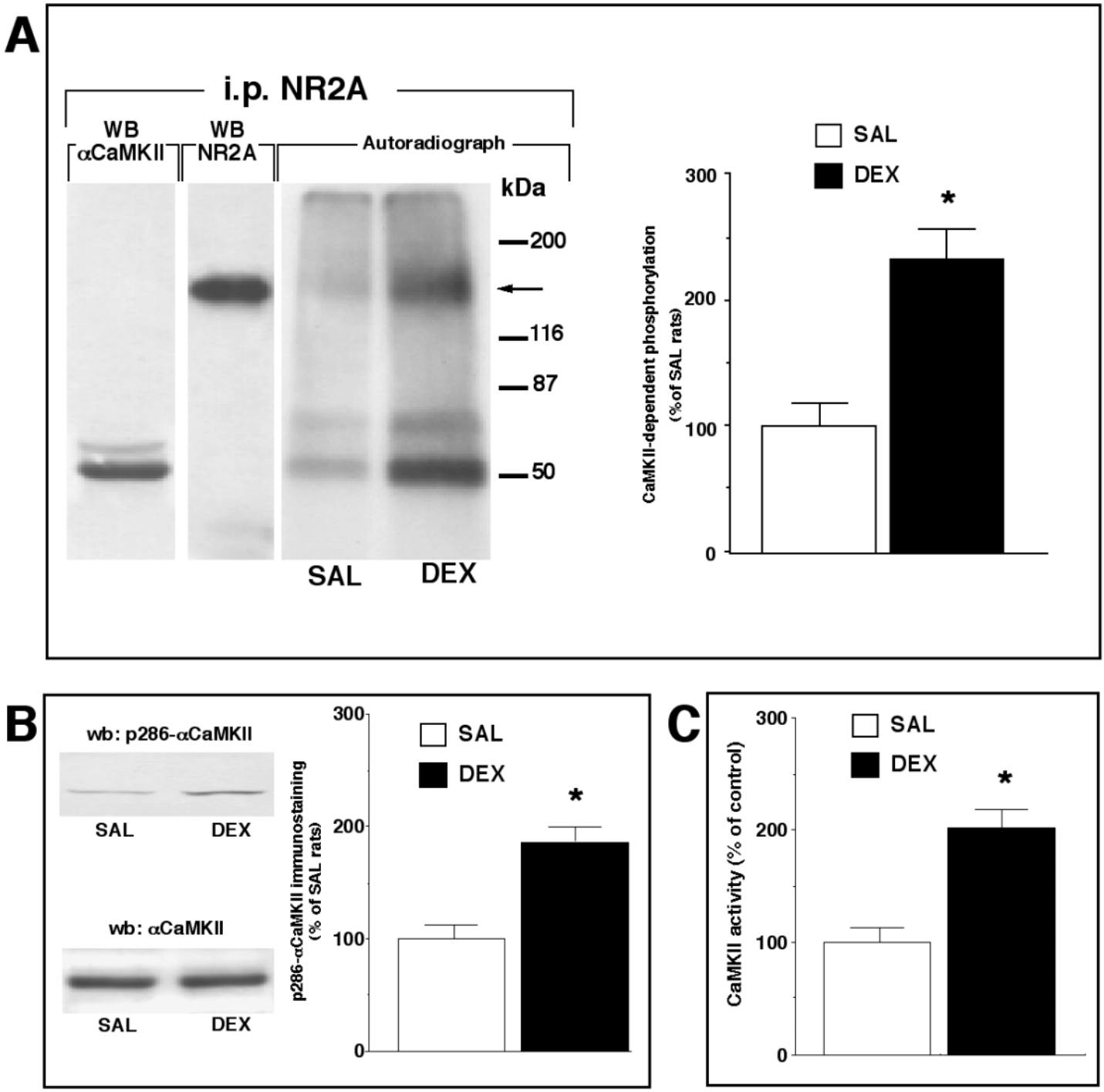

Figure 5. αCaMKII activity and αCaMKII-dependent phosphorylation of NR2 subunit are increased in hippocampal PSD purified from adult rats neonatally treated with DEX. A) Phosphorylated PSD proteins are immunoprecipitated with anti-NR2A. Autoradiograph shows a higher phosphorylation of a 170 kDa (NR2A) and a 50 kDa band (αCaMKII) in DEX as compared with SAL rats (right lanes). αCaMKII and NR2A are identified in the immunoprecipitate by Western blot analysis (left lanes). Data of αCaMKII-dependent phosphorylation are expressed as % of SAL rats and are means ± SE (histogram; n=8 for each group; *P<0.01). **B)** Representative Western blot analysis of p286-αCaMKII and αCaMKII in hippocampal PSD from DEX and SAL rats. Data of p286-αCaMKII immunostaining are expressed as % of SAL rats and are means ± SE (n=8 for each group; *P<0.01). There was no difference in αCaMKII protein levels. **C)** PSD-associated αCaMKII is more active in DEX than in SAL rats. Histogram represents in vitro PSD-associated αCaMKII activity. Data are % of SAL (means±SE; *P<0.01).

Fig. 6

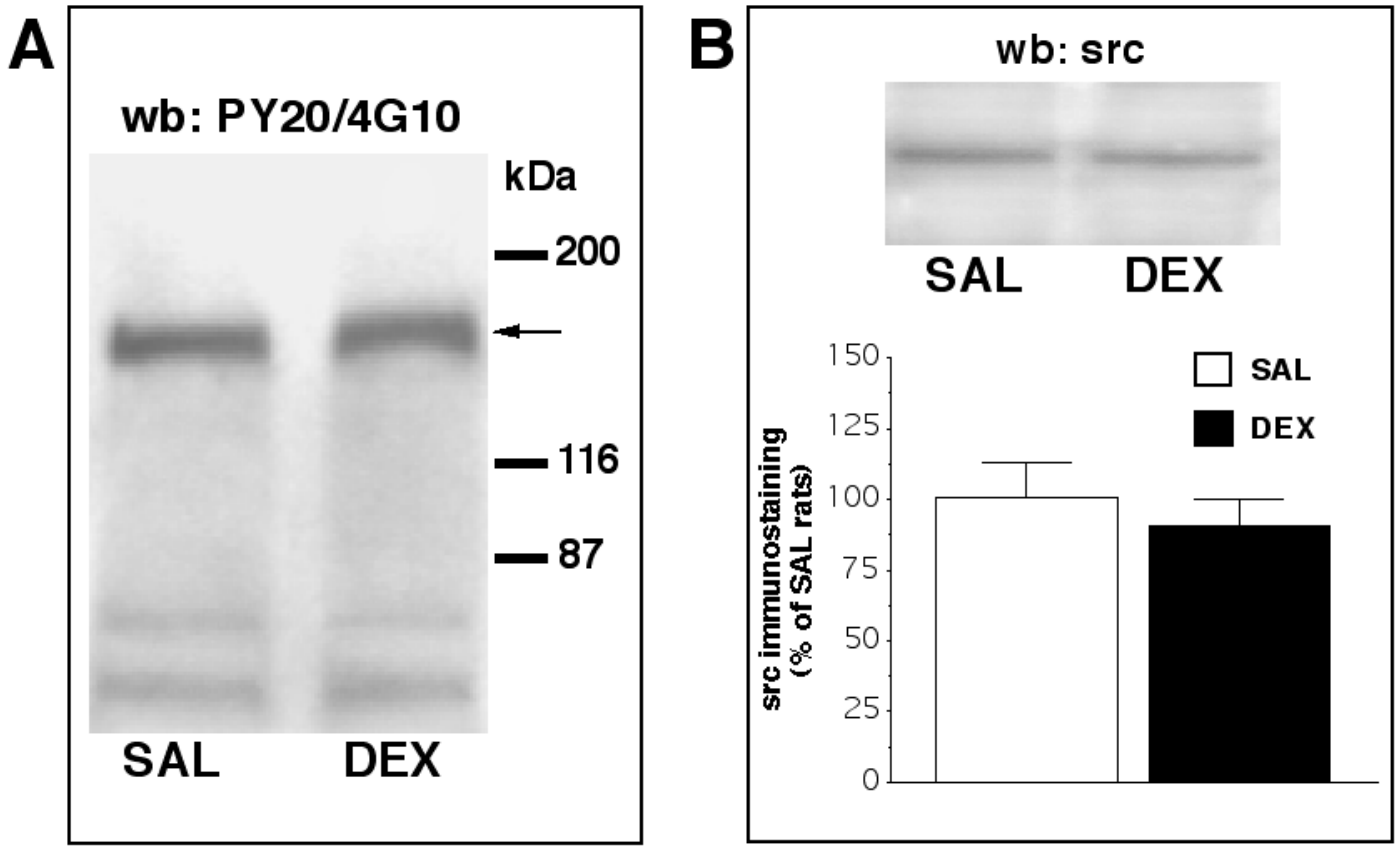

Figure 6. No effect of neonatal DEX treatment on tyrosine phosphorylation in PSD purified from adult rats. **A)** Western blot analysis of phosphotyrosine (PY20/4G10) in hippocampal PSD from SAL and DEX rats. A protein band of ~170 kDa, corresponding to NR2 subunit, is indicated by the arrowhead. **B)** Representative Western blot analysis of src tyrosine kinase in hippocampal PSD from SAL and DEX rats. Data in the histogram are % of SAL immunoreactivity (means±SE; **P*<0.01).