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Acute 19-nortestosterone transiently suppresses hippocampal MAPK pathway and the phosphorylation of the NMDA receptor

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**Abstract**

High doses of anabolic androgenic steroid are associated with changes in personality, e.g. increased aggression and irritability, behavioural changes that may be linked to structural changes in the hippocampus. In this *in vivo* study we demonstrate acute effects of a single injection of 19-nortestosterone on proteins that play a major role in molecular plasticity at synaptic connections. The steroid rapidly and transiently decreased total and phosphorylated NMDA receptor GluN2B subunit levels and phosphorylated extracellular signal-regulated kinase 1 in rat hippocampal synaptoneurosomes. Pretreatment with the androgen receptor antagonist flutamide prevented these effects suggesting an androgen receptor mediated mode of action. However, flutamide alone stimulated the phosphorylation of both extracellular signal-regulated kinase 1 and 2. EphrinB2 and phosphorylated translation initiation factor 4E, two proteins that act on synaptic plasticity through NMDA receptor and/or mitogen-activated protein kinase pathways, were not affected by any of the treatment regimens. This study demonstrates rapid *in vivo* effects of an anabolic androgenic steroid on two key elements in hippocampal synaptic plasticity.

## 1. Introduction

Research during the past two decades has presented evidence that gonadal steroids can modulate neuronal activity in the hippocampus (for review see [1]), a brain structure that plays a vital role in cognition and mood. Estrogens have a well-documented impact on hippocampal neurophysiology and recently it has become clear that androgens exert similar effects although the underlying mechanisms remain relatively unexplored. Androgenic receptors (AR) are present in the hippocampus [2] and several studies have demonstrated that androgens can enhance and restore cognitive performance (for review see [3]).

Classical steroid action regulating gene expression is mediated through nuclear receptors and the effects typically occur in days, however, today it is known that steroid-induced changes in neuronal cell signalling and behaviour can take place within minutes or hours [4]. Such rapid action are suggested to engage extranuclear mechanisms e.g. modulation of GABA [5] and NMDA receptors (NMDAR) [6] or sex steroid receptors located in the cell membrane [7,8], often involving posttranslational modifications of synaptic components, e.g. phosphorylation of NMDARs and extracellular signal-regulated kinase (ERK). For example androgens have been shown *in vitro* to regulate the phosphorylation of ERK within minutes [9,10].

A growing amount of evidence from studies in humans shows that abuse of high doses of anabolic androgenic steroids (AAS) induce changes in mood and personality [11] that are often expressed as increased irritability and bad temper [12]. Increased aggression has also been demonstrated in rats [13]. In addition, androgens are associated to decreased anxiety in rodents [14,15], an effect that may occur within minutes [16]. The underlying molecular mechanism for these effects is still not well known, however, phosphorylation of hippocampal NMDAR and ERK has been linked to fear and anxiety. For example, a reduction in their phosphorylation state are shown to impair the retrieval of contextual fear memory in mice [17,18].

In an earlier report we studied the effects of a single or repeated high doses (daily for 14 days) of an AAS on selected elements in hippocampal synaptoneurosome. This preparation is often used in studies of the regulation of proteins and downstream signalling pathways in close proximity to the synapse. The (sub)chronic administration of AAS did not alter any of the protein or phosphoprotein studied. However, twenty-four hours after the single dose, AAS induced an increase in the phosphorylation of the NMDAR subunits GluN2A and GluN2B and ERK1/2 [19]. Such modifications are considered to constitute important regulatory steps in the cascade underlying synaptic plasticity. To investigate the rapidity of these responses to AAS we here describe the acute effect of 19-nortestosterone (19NT) in hippocampal synaptoneurosome two and six hours after a single systemic injection of the steroid. The AR antagonist flutamide was included in order to clarify whether classical ARs were involved. In search for proteins engaged in the phosphorylation of the NMDAR and ERK, we also studied the eukaryotic initiation factor 4E (eIF4E). This factor, known to be activated by ERK, is essential for mRNA translation and as such considered to be a marker and regulator of

synaptodendritic protein synthesis underlying structural plasticity [20]. Furthermore, we determined the protein levels of ephrinB2, an ERK regulated synaptic component that can stimulate the phosphorylation of GluN2B at Y1472 through the src family kinase Fyn [21,22]. The results show that 19NT acutely and transiently decreases phosphorylated GluN2B and ERK through a glutamate sensitive pathway.

## 2. Materials and methods

The study was approved by the local experimental animal committee. Male Sprague-Dawley rats (Alab, Sollentuna, Sweden), weighing 250 to 275 g, were housed in air-ventilated rooms (humidity 50-60%, temperature 22-24°C) under a 12 h-dark/12 h-light cycle with food and water provided ad libitum. The animals were randomly divided into five groups (assigned oil+oil, oil+19NT, oil+19NT 6 h, Flu+oil, Flu+19NT), each consisting of six rats. Three groups of animals, oil+oil, oil+19NT and oil+19NT 6 h, were daily treated with 250 µl peanut oil (Apoteket AB, Sweden) injection during 5 days. The other two groups Flu+oil and Flu+19NT received daily flutamide (Sigma, F9397) administration during 5 days (15 mg/kg/day in 250 µl peanut oil). Five hours after the last injection, the animals in the groups of oil+19NT, oil+19NT 6 h and Flu+19NT received a single dose of 19NT (Sigma, N7252) (3 mg/kg dissolved in 10% ethanol in peanut oil). Rats in the groups of oil+oil and Flu+oil were injected with 50 µl 10% ethanol in peanut oil. The animals were decapitated 2 h after the last injection except for the rats of the oil+19NT 6 h group. Animals of the oil+19NT 6 h group were killed 6 h after the administration of the steroid. Right and left hippocampus was rapidly dissected out on ice, using a rat brain matrix (Activational System Inc., Mortella Drive Warren, MI, USA), and placed on dry ice. The tissues were kept at -80°C until further processing

### 2.2. Synaptoneurosomes preparation

A preparation of rat synaptoneurosomes, enriched for synaptic components, was isolated according to the method of Hollingsworth et al [23]. Using a Teflon pestle homogeniser, individual left hippocampi were homogenised in 10 volumes of ice-cold buffer pH 7.4 (HEPES 50 mM, NaCl 124 mM, NaHCO<sub>3</sub> 26 mM, glucose 10 mM, MgCl<sub>2</sub> 1.3 mM, CaCl<sub>2</sub> 2.5 mM, KCl 3.2 mM, KH<sub>2</sub>PO<sub>4</sub> 1.06 mM, chloramphenicol 0.7 mM, saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>) containing phosphatase inhibitor cocktail 1 & 2 (Sigma, P2850 & P5726) and protease inhibitors (Complete Mini tablets, Roche). The crude homogenate was diluted, mixed with an additional 10 volumes of the ice-cold homogenisation buffer and incubated on ice for 10 min. The preparation was filtered through two layers of 80 µm nylon net filter disks (NY8002500, Millipore) and the filtrates were then passed through a 5µm Durapore<sup>®</sup> membrane (SVLP01300, Millipore). The filtered solutions were centrifuged at 1000 x g for 15 min at 4°C, the pellets were resuspended in 8 volumes of homogenisation buffer and aliquots were kept at -80°C. Protein concentration was determined by use of a commercial kit (BCA Kit, Pierce). The GluN2A and GluN2B subunit proteins were used as markers for the accumulation of synaptic proteins in the synaptoneurosomes preparation. Moreover, the content of ERK1/2, eIF4E and ephrinB2 was determined in the different fractions.

### 2.3. Western blotting

A randomized block design was used for the analysis. Ten or twenty  $\mu\text{g}$  of protein (depending on protein analysed) were resolved on 10% SDS-polyacrylamide gels and transferred to a 0.45  $\mu\text{m}$  nitrocellulose membrane (Hybond<sup>®</sup>-ECL, GE Healthcare). Blots were incubated with specific antibodies recognising the following proteins: ERK1/2 (#9102, 1:1500, New England Biolabs) and p-ERK1/2 (1:3000, #9101, New England Biolabs); p-GluN2A<sup>S1232</sup> (1:10000, #2056, Tocris Bioscience); p-GluN2B<sup>Y1472</sup> (1:3000, SIG-9063, Signet Laboratories); GluN2A (1:40 000, sc-1468, Santa Cruz), GluN2B (1:20000, sc-1469, Santa Cruz); p-eIF4E (1:2500, #9741, Cell Signalling); eIF4E (1:10000, #9742, Cell Signalling); ephrinB2 (1:24000, a generous gift from Dr. Klas Kullander, Dept. of Neuroscience, Uppsala University). The preparations were subsequently incubated with the corresponding secondary horseradish peroxidase (HRP) conjugated antibodies (Santa Cruz). Immunoblots were visualised using standard chemiluminescence reagent or enhanced chemiluminescence reagent (ECL Detection Reagent, GE Healthcare) when appropriate and exposed to Cronex 5 light-sensitive film (Agfa Gevert). When stripped, the filters were washed with a buffer containing 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.8, at 55°C for 20 minutes. The ImageJ 1.37p software (NIH, USA) was used to quantify the band density of the scanned films. Protein quantification, inter-lane uniformity in loading and transfer was verified in two separate western blots where 10  $\mu\text{g}$  protein from each individual biological sample were loaded on 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes. The total protein content of each sample transferred to the membrane was quantified by use of MemCode<sup>®</sup> protein staining (Thermo Fisher Scientific, Rockford, USA).

### 2.4 Biochemical characterization of the synaptoneurosomal preparation

The distribution of the proteins studied was determined in crude homogenate, synaptoneurosomes and supernatant prepared as described in section 2.2. Equal amounts of protein from respective fraction were analysed by western blot as described above (section 2.3).

### 2.5. Real time quantitative PCR

For real time quantitative polymerase chain reaction (qPCR), total RNA was isolated from right hippocampus (TRIzol<sup>®</sup> reagent, Invitrogen) and treated with DNase (Promega M610A). RNA quality was controlled with respect to 28S/18S ratio (Experion Automated Electrophoresis System, RNA StdSens Analysis Kit, Bio-Rad Laboratories) and the absence of genomic DNA was confirmed by PCR. cDNA was synthesised (Superscript III Kit, Invitrogen) according to the manufacturer's protocol. qPCR was performed in an iCycler iQ multicolor real time detection system (Bio-Rad Laboratories) in a reaction mixture consisting of cDNA (corresponding to 25 ng transcribed RNA), 20  $\mu\text{l}$  of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM dNTP, SYBR Green 1:50000, 12.5 nM fluorescein, 0.25  $\mu\text{M}$  of forward and reverse primer and 0.04 U/ $\mu\text{l}$  Taq DNA polymerase. The following conditions were applied for the qPCR reaction; 95°C for 3 min followed by 50 cycles at 95°C for 15 sec, T<sub>a</sub> for 15 sec and 72°C for 30 sec. A melting point curve was recorded for each run to confirm that only one single product was formed. All qPCR experiments were performed in triplicates on individual samples

and negative controls. The analysis of the qPCR experiments was conducted as follows. The cycle threshold (ct) values were obtained from the iCycler 3.0.6 software (Bio-Rad Laboratories) and the triplicate averages were transformed into quantities between 0 and 1, where the highest relative quantity (lowest ct value) was set to 1. To test for the most stable set of house-keeping genes (HKG) as reference we used the method of Vandesompele et al. [24]. Out of 7 genes histone H3,  $\beta$ -actin and  $\beta$ -tubulin were selected using the GeNorm software [24]. The obtained normalisation factors were used for normalisation of GluN2A and GluN2B mRNA levels.

The following primer sequences with the corresponding annealing temperatures ( $T_a$ ) were used: histone H3, ( $T_a$ : 60.0°C): forward 5'-ATT CGC AAG CTC CCC TTT CAG-3', reverse 5'-TGG AAG CGC AGG TCT GTT TTG-3';  $\beta$ -actin, ( $T_a$ : 60.0°C): forward 5'-CAC TGC CGC ATC CTC TTC CT-3', reverse 5'-AAC CGC TCA TTG CCG ATA GTG-3';  $\beta$ -tubulin ( $T_a$ : 60.0°C): forward 5'-CGG AAG GAG GCG GAG AGC-3', reverse 5'-AGG GTG CCC ATG CCA GAG C-3'; GluN2A, ( $T_a$ : 61.0°C): forward 5'-CAG CAG CAG GCC ACA GTT ATG-3', reverse 5'-AGT CTC GGT AGC CAG GGG AG-3'; GluN2B, ( $T_a$ : 62.8°C): forward 5'-GTG AAC AAG ATC CGC AGT ACC ATC-3', reverse 5'-CTT AGA GTC GCC ATC GTC CAG AG-3'.

## 2.6. Immunoprecipitation and detection of the androgen receptor

Immunoprecipitation was performed using a commercially available kit (Dynabeads<sup>®</sup> Protein A, Invitrogen). As an AR positive control for we used LNCaP cells. The synaptoneuroosomes or LNCaP cells were treated with lysis buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, supplemented with phosphatase inhibitor cocktail 1 & 2 (Sigma, P2850 & P5726) and protease inhibitors (Complete Mini tablets, Roche) at a ratio of 1:2) for 30 min at 4°C. After a subsequent centrifugation at 18000 x g for 10 min the clear lysate was collected. For immunoprecipitation AR antibody (5  $\mu$ g, sc-815, SantaCruz) was bound to the beads and subsequently incubated with lysates for 15 min at room temperature. After three washes the antigen was eluted and denatured by heat. The eluates were loaded onto an 8% SDS-polyacrylamide gel and the AR protein was identified by western blot as described in section 2.3. Blots were incubated with a specific AR antibody (PG-21) (1:50000; Millipore, #06-680) followed by incubation with anti-rabbit secondary HRP conjugated antibody (Bio-Rad, #172-1019). The immunoblots were visualised as described in section 2.3.

## 2.7. Data analysis and statistics

For the determination of phosphorylated proteins the ratio of phosphorylated to total protein was calculated for each individual animal. The mean values from the treated groups were expressed as a percentage of the mean value from the control group. Data are expressed as means  $\pm$  SEM. Differences between the groups of animals were calculated using one-way ANOVA. When the ANOVA reached a significant level ( $P < 0.05$ ) a Fischer's PLSD *post-hoc* test was computed. Statistical analysis was performed in StatView software (StatView 5.0.1, SAS Institute, 1998).  $P < 0.05$  was considered as significant level.



### 3. Results

#### 3.1. Protein profile of the hippocampal synaptoneurosome preparation

The different fractions of the hippocampal synaptoneurosome preparation (crude homogenate, synaptoneurosomes and supernatant) were analysed for their content of GluN2A, GluN2B, ERK1/2, eIF4E and ephrinB2 (Fig. 1). The NMDAR subunits showed as expected an accumulation in the synaptoneurosomal fraction. The levels of ERKs and eIF4E were reduced in synaptoneurosomes while the ephrinB2 concentrations did not vary notably between the three fractions.

#### 3.2. Validation of the western blot analysis

The determination of all proteins was performed by western blot analysis. MemCode<sup>®</sup> protein staining was used for the validation of protein quantification and inter-lane uniformity in protein loading and transfer, yielding a coefficient of variance (CV) of 7.2 %.

#### 3.3. The effect of 19NT on ERKs and the impact of flutamide

The phosphorylation of ERK1 (Fig. 2A) and ERK2 (Fig. 2B) were significantly altered by the treatment with flutamide and/or 19NT ( $F_{3,18} = 12.8$ ,  $P < 0.001$ ;  $F_{3,18} = 6.41$ ,  $P < 0.01$ , respectively, one-way ANOVA). The post-hoc test (Fisher's PLSD) revealed a significant decrease of the ERK1 phosphorylation and a tendency to a decrease of phosphorylated ERK2 levels two hours after the administration of 19NT (p-ERK1;  $66.1 \pm 6.8\%$ ,  $P < 0.01$ , p-ERK2;  $84.2 \pm 4.7\%$ ,  $P = 0.07$ ). When the rats were treated with flutamide prior to the administration of 19NT, p-ERK1 levels were not only restored but were significantly higher compared to the control group ( $129 \pm 7.5\%$ ,  $P < 0.001$  compared to oil+19NT;  $P < 0.05$  compared to oil+oil). Regarding p-ERK2, the latter treatment regimen resulted in significantly higher levels compared to animals treated with oil+19NT ( $118 \pm 6.7\%$ ,  $P < 0.01$ ). Surprisingly, the five-days pretreatment with flutamide alone resulted in stimulation of both ERK1 and ERK2 phosphorylation ( $123.8 \pm 10.5\%$ ,  $P < 0.05$ ;  $129.1 \pm 7.4\%$ ,  $P < 0.05$ , respectively).

#### 3.4. The effect of 19NT on NMDA receptor subunits and the impact of flutamide

The one-way ANOVA analysis of the effects of the flutamide and/or 19NT treatment on p-GluN2B<sup>Y1472</sup> levels in rat hippocampal synaptoneurosomes revealed significant differences between the groups of rats ( $F_{3,18} = 4.74$ ,  $P < 0.05$ ). Post hoc test (Fisher's PLSD) showed that p-GluN2B<sup>Y1472</sup> was significantly decreased ( $63 \pm 10\%$ ,  $P < 0.05$ ) two hours after the administration of 19NT when compared to control animals ( $100 \pm 10\%$ ) (Fig. 2C). While pretreatment with the androgen receptor antagonist flutamide alone had no statistically significant effect on p-GluN2B<sup>Y1472</sup>, it completely blocked the effect induced by 19NT at 2 h ( $118.6 \pm 18.0\%$ ,  $P < 0.01$ ) (Fig. 2C). Significant differences between the groups of animals were also found for total GluN2B protein ( $F_{3,17} = 3.86$ ,  $P < 0.05$ , one-way ANOVA) (Fig. 2D). The levels of GluN2B tended to be reduced two hours after the injection of 19NT, an effect that almost reached a significant level ( $87.2 \pm 4.9\%$ ,  $P = 0.06$ , Fisher's PLSD). Flutamide pretreatment resulted in significantly higher

levels of GluN2B compared to this latter group of rats ( $108.1 \pm 2.5\%$ ,  $P < 0.01$ , Fisher's PLSD). The levels of p-GluN2A<sup>S1232</sup> and GluN2A were not affected in any of the animal groups (Fig. 4A-B).

### 3.5. Time-dependent alterations of GluN2B and ERK phosphorylation after 19NT administration

To investigate if the time of exposure influenced the effect of 19NT on NMDAR and ERK phosphorylation, we included a group of animals that were sacrificed six hours after the injection of 19NT. The results (Fig 3A-D) showed a significant impact of time on p-GluN2B<sup>Y1472</sup> and p-ERK1 levels ( $F_{2,14} = 7.64$ ,  $P < 0.01$ ;  $F_{2,14} = 6.32$ ,  $P < 0.05$ , respectively, one-way ANOVA). The decrease in phosphorylation of GluN2B<sup>Y1472</sup>, ERK1 and ERK2 detected two hours after androgen administration was restored after six hours ( $111 \pm 5.5\%$ ,  $P < 0.01$ ;  $121 \pm 16\%$ ,  $P < 0.05$ ;  $112 \pm 9.8\%$ ,  $P < 0.05$ , respectively, Fisher's PLSD post-hoc test). Although phosphorylated protein levels six hours after 19NT administration tended to be higher compared to control levels, they never reached significant levels. GluN2B levels were also transiently decreased (one-way ANOVA ( $F_{2,13} = 5.57$ ,  $P < 0.05$ ) with a significant reduction two hours after 19NT administration ( $87.2 \pm 4.9\%$ ,  $P < 0.05$ ) and restored levels at six hours ( $102.6 \pm 3.3\%$ ,  $P < 0.01$ )

### 3.6. The effect of 19NT and flutamide on p-eIF4E and ephrinB2

Western blot analysis of the hippocampal synaptoneurosomal fraction revealed that the treatment regimens neither affected the phosphorylated form of the marker for synaptodendritic protein synthesis eIF4E nor the protein amount of the Eph receptor ligand ephrinB2 (Fig. 4).

### 3.7. Determination GluN2A, GluN2B and ephrinB2 mRNA levels

Quantitative real time PCR was performed to investigate if the 19NT-induced decrease in GluN2B protein levels at two hours reflected downregulated mRNA expression levels. Also the transcript for GluN2A was determined. However, neither GluN2B nor GluN2A mRNA expression was affected by 19NT or sub-chronic flutamide administration (Tab.1).

### 3.8. Detection of AR in the synaptoneurosomal preparation and LNCaP cells

To investigate the presence of AR in our hippocampal synaptoneurosomal fraction, western blot analysis was performed on immunoprecipitate from synaptoneurosomes or whole cell extract from the AR containing human prostate LNCaP cell line. Figure 5 displays a single immunoreactive entity in synaptoneurosomes. The molecular mass (approx. 110 kD) was found to be similar to that of the AR detected in LNCaP cells.

## 4. Discussion

In this study we determined the acute effect of a single systemic dose of the AAS 19NT on selected key elements in hippocampal synaptoneurosomes. This synaptic subcellular fraction is suitable for studies of the regulation of proteins at synapses. As expected,

NMDAR subunits are accumulated in this preparation while ERKs and eIF4E are reduced (Fig. 1). Nevertheless, both ERKs and eIF4E play important roles in protein synthesis locally at synapses [25,26]. EphrinB2 at its synaptic localisation, is believed to be involved in synaptic plasticity [27], however in our fractions it is evenly distributed supporting the findings that it is also present in neuronal cell bodies in rodent hippocampus [28].

Two hours after 19NT administration the phosphorylation state of the NMDA receptor subunit GluN2B was decreased while that of the GluN2A subunit was unaffected. Simultaneously, p-ERK1 (and p-ERK2 to some extent) declined in a comparable manner. These declines were blocked when the AR antagonist flutamide was administered daily during five days prior to the acute dose of 19NT. This suggests an AR mediated mode of action. Six hours after the injection of 19NT, the levels of p-GluN2B<sup>Y1472</sup> and p-ERK1 were restored. Moreover, the antiandrogen flutamide exerted its own effects by significantly increasing p-ERK1 and p-ERK2 levels.

The published effects of androgens and antiandrogens on cell signalling are in part inconsistent and the underlying mechanisms remain unclear. For example, flutamide or its active metabolite hydroxyflutamide mimic rather than inhibit androgen-induced phosphorylation of ERK in prostate [29,30] and breast cancer cell lines [31]. It is speculated that this effect is mediated through a non-genomic pathway involving extranuclear ARs which, in fact have been visualised in rat hippocampal axons and dendrites by immunocytochemistry [32]. Since these morphological components are enriched in the synaptoneuroosomes, they may represent the origin for the immunoprecipitated ARs that we detect in this preparation. In both intact and AR deficient mice, DHEA and flutamide increase hippocampal dendritic spine synapse number [33], a process that is known to depend on NMDAR regulated MAPK signalling [34]. Conversely, a differential effect of androgens and flutamide on ERK activation has also been observed. In glial C6 cells, dihydrotestosterone (DHT) increased ERK phosphorylation in a flutamide sensitive manner while membrane-impermeable DHT-BSA decreased p-ERK, an effect that flutamide was unable to block [9]. The authors of that study suggested that nuclear AR mediated the stimulatory effect while a distinct membrane AR was responsible for the decline in p-ERK.

Our study was performed on intact male rats, which comprise an *in vivo* system much different from isolated cells. Interpreting the effect of 19NT alone, and 19NT in combination with flutamide pretreatment, it appears that the steroid acts through flutamide sensitive ARs. However, the increase in p-ERK seen after the administration of flutamide is difficult to explain. Subchronic AR antagonist treatment is considered to block nuclear ARs and to decrease AR gene expression in the hippocampus [2]. Since the rats used in our study were gonadally intact, a normal testosterone production could be assumed. If androgens exert a constitutive suppression of ERK phosphorylation, a block of the basal endogenous androgen tonus by AR antagonists may result in elevated p-ERK levels in the respective group of animals. Regarding 19NT, the effects appear after two hours, which is a relatively short period of time for the steroid to act via its nuclear receptor in a classical genomic way. The decreased phosphorylation induced by 19NT is

in line with the previously published effect of membrane-impermeable DHT-BSA in C6 cells [9] and may thus reflect an involvement of a membrane-associated AR receptor. However, if the two effects mediated by the different targets are opposing why was 19NT not able to reduce the flutamide-induced increase in p-ERK1? Possible explanations may be that the steroid concentration was too low or that the effects induced by 19NT alone are also mediated through classical ARs.

The 19NT-induced decrease in p-GluN2B<sup>Y1472</sup> discussed above was paralleled by a decline in the levels of GluN2B. This is consistent with the notion that Fyn kinase mediated phosphorylation of GluN2B<sup>Y1472</sup> regulates the localisation of GluN2B containing NMDARs to the synaptic membrane [35,36]. To rule out that the altered GluN2B level was due to decreased GluN2B transcription, we determined its mRNA expression by qPCR. The observation that GluN2B mRNA levels were unchanged argues against transcriptional regulation in our study. Likewise, translation does not seem to be affected either, since there was no change in the levels of the activated (phosphorylated) form of the translation initiation factor eIF4E that regulates the translation of most mRNAs [37]. Thus, it appears that the depleted GluN2B protein levels observed in the present study are a consequence of increased internalisation due to decreased phosphorylation of GluN2B<sup>Y1472</sup>. Nateri et al. [21] suggests that MEK1 overexpression in mice leads to elevated hippocampal GluN2B protein levels through increased ERK phosphorylation and subsequent activation of eIF4E. In that study, GluN2B levels were not associated with those of phosphorylated GluN2B<sup>Y1472</sup>. We observe analogous changes in the levels of p-ERK, p-GluN2B<sup>Y1472</sup> and GluN2B although p-eIF4E remained unchanged. These findings indicate a connection between NMDAR regulation and ERK activation in the rodent hippocampus. It is known that phosphorylation of GluN2B<sup>Y1472</sup> affects NMDAR activity [38], which in turn has been shown to regulate the phosphorylation of ERK in the synaptosomal compartment [39]. This mechanism is believed to represent a link in dendritic local protein synthesis essential for synaptogenesis. Although androgens are reported to stimulate synaptogenesis [40], our results do not support an engagement of eIF4E in the synaptic fraction at least not within the time frame tested in the present investigation. Looking up-stream of NMDAR regulation, ephrinB2 is a protein that stimulates the phosphorylation of GluN2B at Y1472 through Fyn kinase [21,22]. Moreover, it is also known to regulate synaptic plasticity [27]. We were however not able to detect any changes in ephrinB2 protein levels suggesting that the regulation of GluN2B<sup>Y1472</sup> in this study, depends on alternative pathways.

Interestingly, phosphorylation of the GluN2B subunit at Y1472 and ERK in the hippocampus has been linked to fear and anxiety. A reduction of the phosphorylation state in the hippocampus is shown to impair the retrieval of contextual fear memory in mice [17,18]. Androgens are reported to affect mood and while long-term treatment with AAS is anxiogenic in rats [41,42], testosterone has been shown to rapidly (after 30 min) decrease anxiety in mice [16]. Moreover, male rats with recent sexual experience show increased hippocampal testosterone and decreased anxiety-like behaviour [43]. In humans, a single dose of testosterone reduces fear-potentiated startle when tested four hours after the administration [44]. In view of our results showing that AAS acutely (after

two hours) decrease hippocampal p-GluN2B<sup>Y1472</sup> and p-ERK1, it is interesting to note reports on criminals that prime themselves with AAS to become more calm prior to a criminal act [45]. This may thus represent a direct application on a fast steroid-induced effect on NMDAR and ERK phosphorylation. Furthermore, flutamide has been demonstrated to increase anxiety-like behaviour in rats [46] and in humans [47], an effect that is in accordance with increased p-ERK levels as seen in the group of rats treated with flutamide alone.

An alternative non-genomic pathway for the action of 19NT could be a direct or indirect interaction with ion channels. The function of ion channels such as NMDA and GABA receptors can be rapidly modulated by neurosteroids through allosteric interactions at receptor sites [48]. AAS and neurosteroids show structural similarity, yet they can distinctively modulate the GABA<sub>A</sub> receptor due to differences at critical moieties [49]. With respect to the NMDAR we have previously shown that the neurosteroids, e.g. pregnenolone sulfate and pregnanolone sulfate, differentially interacts with the GluN2B subunit via an allosteric modulation [6,50]. We know that chronic administration (21 days) of 19NT to rats does not affect that modulation of the receptor subunit (unpublished data). However, studies addressing an acute impact of AAS in that context have not been conducted. In view of the study by Chen et al.[51], demonstrating that pregnenolone sulfate rapidly stimulates the phosphorylation of ERK and GluN2B when applied to hippocampal slices, and the fact that structurally different neurosteroids often display differential effects, one could speculate on a mechanism where 19NT, or metabolites thereof, act oppositely to pregnenolone sulfate.

In summary, a single injection of the androgenic steroid 19NT acutely and transiently decreased the phosphorylation of the GluN2B subunit of the NMDA receptor and ERK1 in rat hippocampal synaptoneurosome. Pretreatment with flutamide abolished the effect indicating an AR mediated mode of action, however, flutamide displayed an effect of its own increasing both p-ERK1 and p-ERK2. The results show that androgens have a fast *in vivo* effect on major players in structural synaptic plasticity although the markers for synaptogenesis tested here were not affected by the treatment. Whether acute AAS decreases anxiety-like behaviour in the rat, as indicated by the effect on p-GluN2B<sup>Y1472</sup> and p-ERK1, is an issue for further investigation.

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**Figure legends:**

Fig. 1

Protein profile of the hippocampal synaptoneurosome preparation. Immunoblots of different fractions (crude homogenate, synaptoneurosome and supernatant) were compared for their content of GluN2A (A), GluN2B (B), ERK1 (C), ERK2 (D), ephrinB2 (E) and eIF4E (F). Representative immunoblots for each fraction are shown on top of each diagram. The values are means  $\pm$  SEM of two independent Western blot analyses.

Fig. 2

The effect of subchronic (5 d) flutamide and/or acute (2 h) 19NT treatment on the p-ERK1 (A), p-ERK2 (B), p-GluN2B<sup>Y1472</sup> (C) and GluN2B (D) in rat hippocampal synaptoneurosome. Representative immunoblots for each group of rats are shown on top of each diagram. Data is expressed as mean  $\pm$  SEM of two independently conducted Western blot analyses. #  $P = 0.06$ , \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  (one-way ANOVA followed by Fisher's PLSD post hoc test).

Fig.3

The influence of time on the effects of 19NT on synaptoneurosomal p-ERK1 (A), p-ERK2 (B), p-GluN2B<sup>Y1472</sup> (C) and GluN2B (D) in male rat hippocampus. Representative immunoblots for each group of rats are shown on top of each diagram. The values are means  $\pm$  SEM of two independently conducted Western blot analyses. \*  $P < 0.05$ , \*\*  $P < 0.01$  (one-way ANOVA followed by Fisher's PLSD post hoc test).

Fig.4

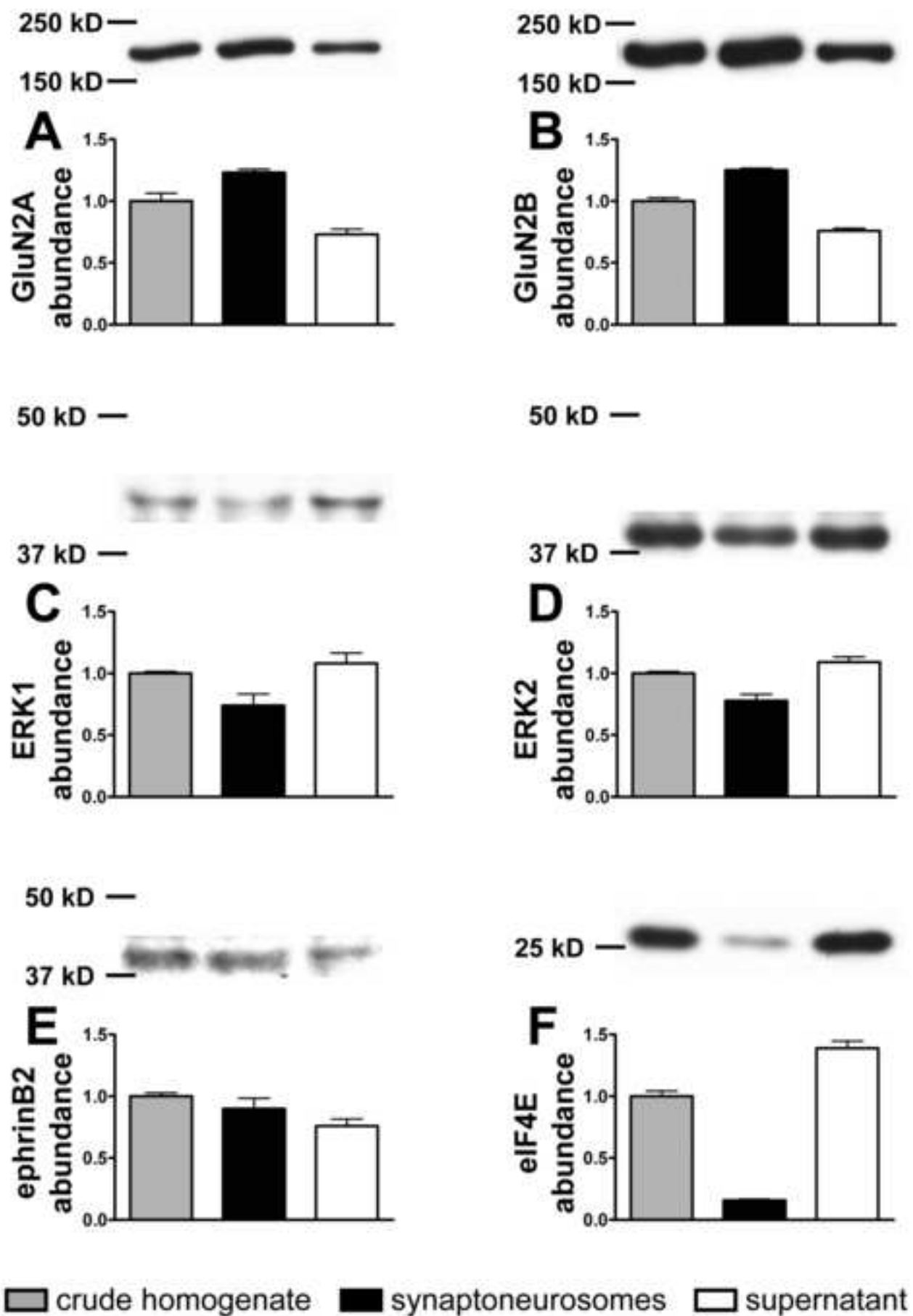
Diagrams show the effect of subchronic (5 d) flutamide and/or acute (2 h or 6 h) 19NT treatment on GluN2A (A), p-GluN2A<sup>S1232</sup> (B), ephrinB2 (C) and p-eIF4E (D) in rat hippocampal synaptoneurosome. Representative immunoblots for each group of rats are shown on top of each diagram. The values are means  $\pm$  SEM of two independently conducted Western blot analyses. One-way-ANOVA analysis did not show any significant variation between the different treatments.

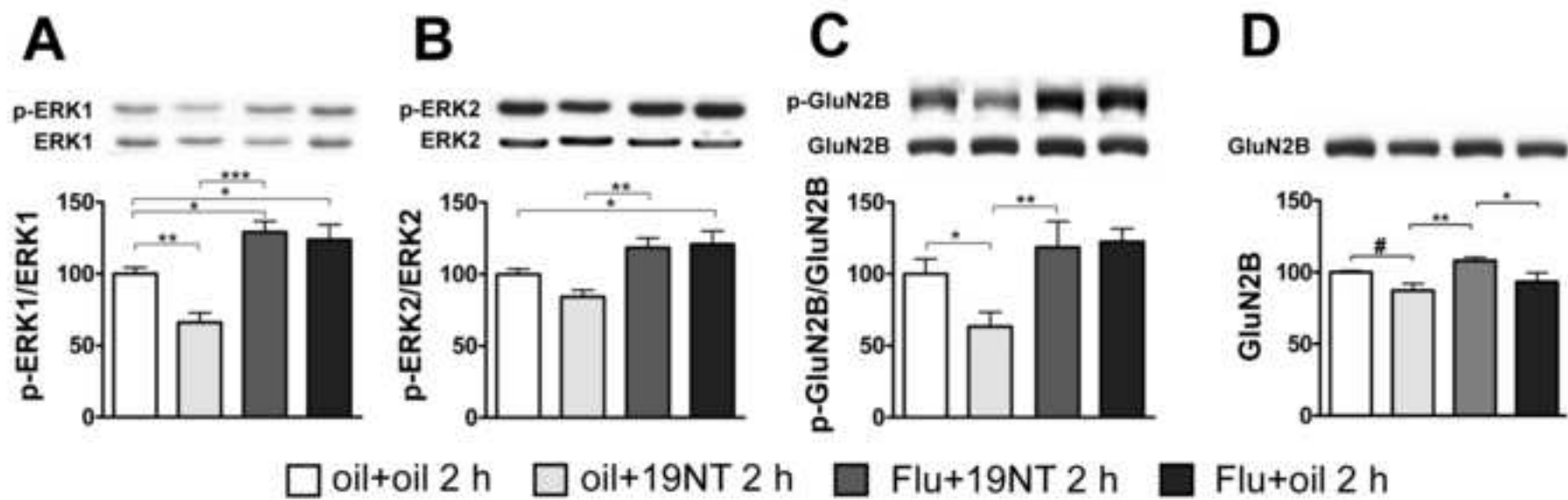
Fig.5

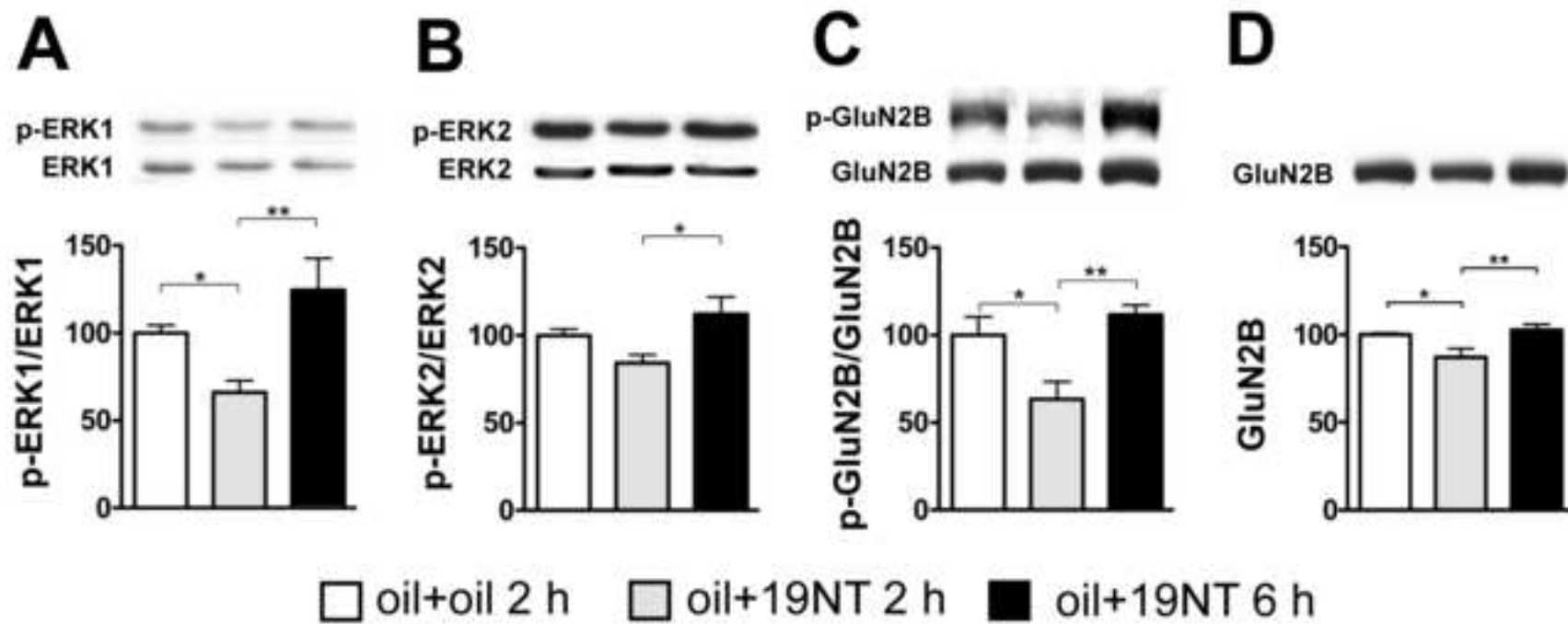
Immunoprecipitation and detection of androgen receptor (AR). This analysis shows the presence of AR protein (~110 kD) in hippocampal synaptoneurosome (SNS) of the male rat. AR is known to be expressed in the total protein extract of human prostate carcinoma cell line (LNCaP) which served as the positive control.

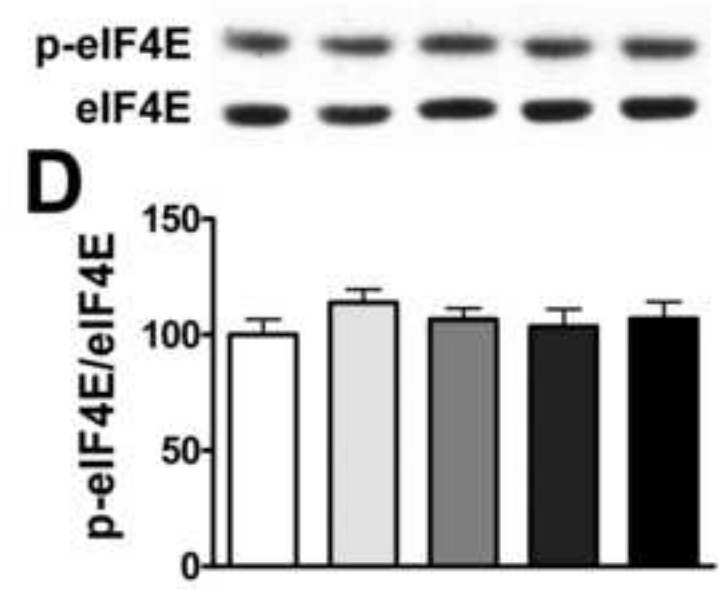
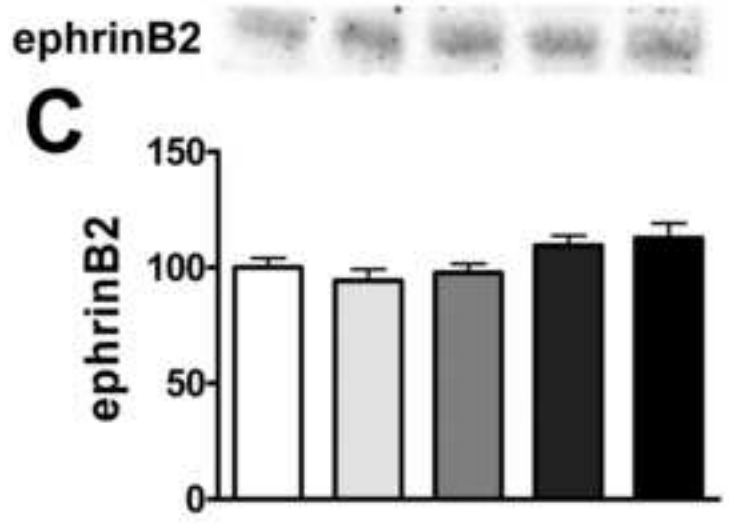
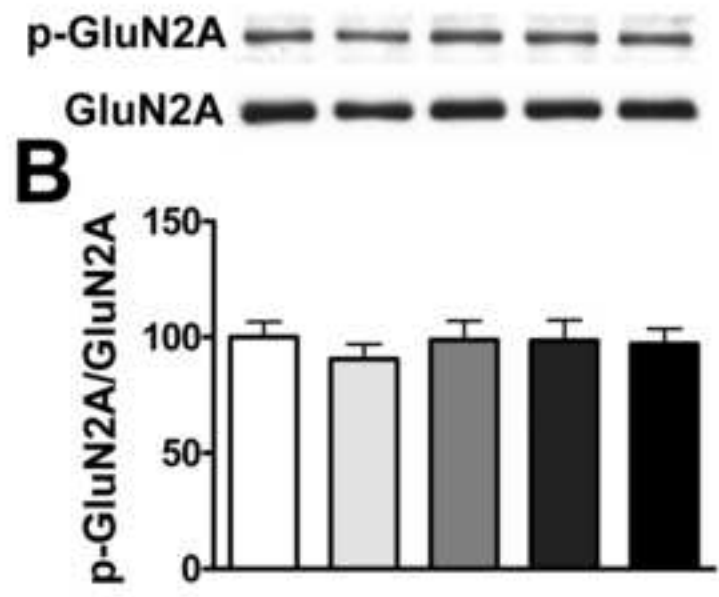
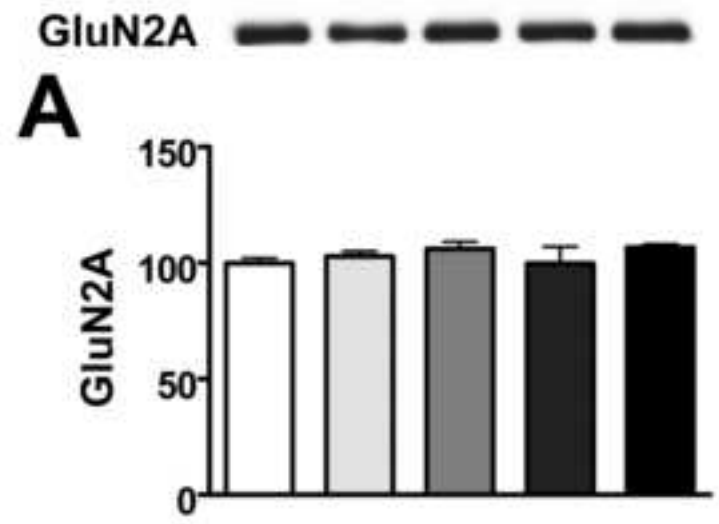
Tab. 1

mRNA expression of NMDA receptor subunits GluN2A and GluN2B in rat hippocampal tissue. The data are presented as mean  $\pm$  SEM. One-way-ANOVA analysis did not reveal any significant variation between the different treatments.



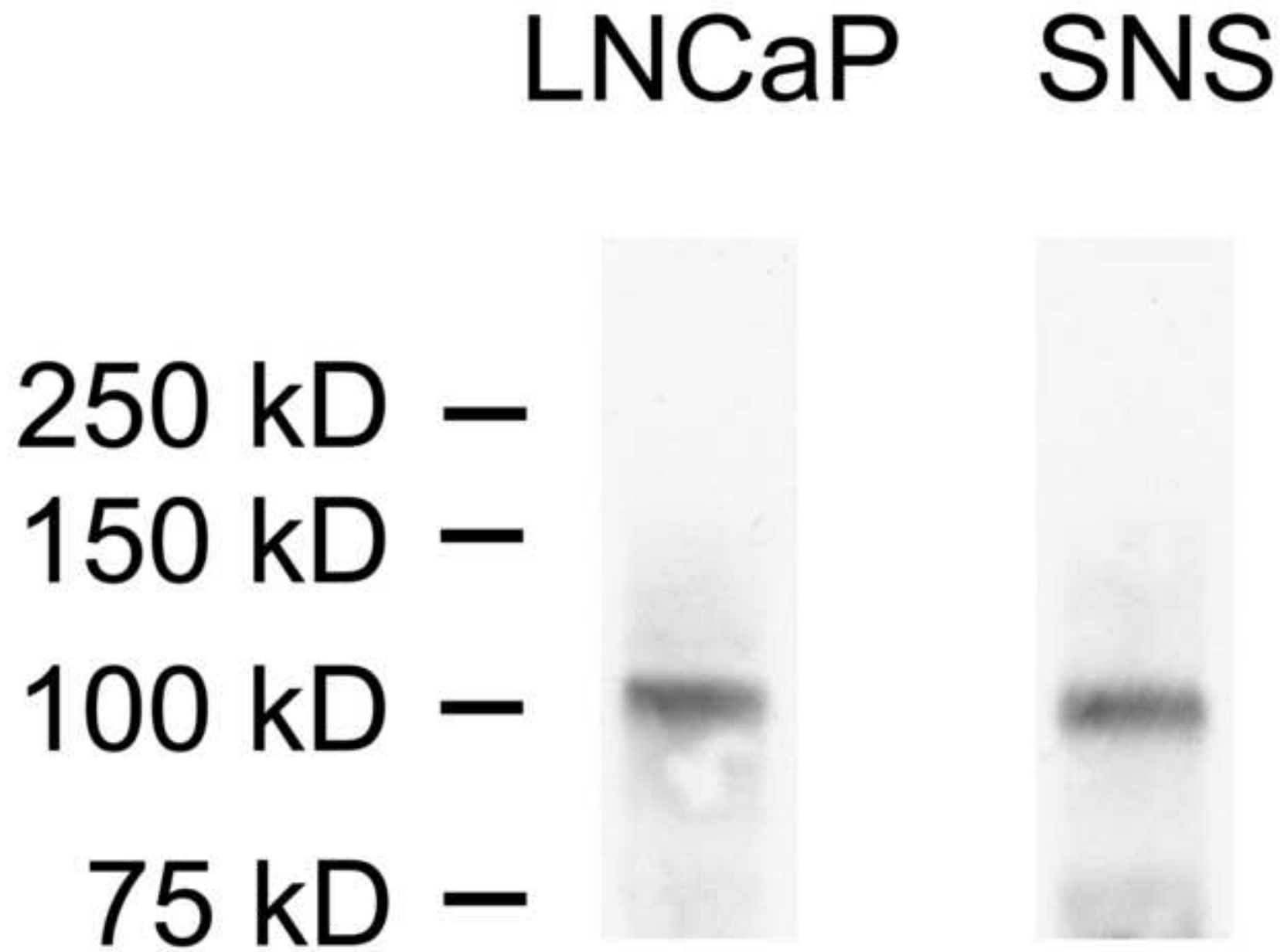






oil+oil 2 h  
  oil+19NT 2 h  
  Flu+19NT 2 h  
  Flu+oil 2 h  
  oil+19NT 6 h

trip





**Table 1 - mRNA expression levels of the NMDA receptor subunit GluN2A and GluN2B in rat hippocampus**

gene	Treatment				
	oil+oil 2 h	oil+19NT 2 h	FLU+19NT 2 h	FLU+oil 2 h	oil+19NT 6 h
GluN2A	100.0 ± 6.7	101.0 ± 4.7	100.2 ± 6.3	103.7 ± 7.0	91.3 ± 4.4
GluN2B	100.0 ± 3.1	103.9 ± 7.5	102.9 ± 3.5	112.8 ± 4.2	104.0 ± 4.2