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Steroid requirements for regulation of the $\alpha 4$ subunit of the GABA_A receptor in an *in vitro* model

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Abstract

The $\alpha 4$ subunit of the GABA_A receptor (GABAR) has relatively low expression in the CNS, but is increased *in vivo* following 48 h administration of the GABA-modulatory steroid 3 α -OH-5 α [β]-pregnan-20-one (THP or [allo]pregnanolone) to female rats. The purpose of the following study was to determine the optimal conditions for steroid-induced upregulation of $\alpha 4$ expression in an *in vitro* model. To this end, we used the IMR-32 cell, a neuroblastoma cell line, which normally expresses $\alpha 4$ mRNA at low levels. In undifferentiated IMR-32 cells, 48 h administration of THP increased $\alpha 4$ expression when ambient THP levels were reduced by the 5 α -reductase blocker 4MA, suggesting that the background steroid milieu affects steroid regulation of this subunit. Following neuronal differentiation in serum-free medium, 48 h THP treatment significantly increased $\alpha 4$ expression two-fold following application of nerve growth factor (NGF) suggesting that development of neuronal processes facilitates this effect of the steroid. In the absence of NGF treatment, combined administration of 17 β -estradiol (E₂) plus THP also increased $\alpha 4$ expression to a similar extent as THP following NGF treatment. In addition, E₂ alone effectively increased $\alpha 4$ expression to maximal levels following NGF treatment. In contrast, neuronal differentiation in the absence of serum deprivation did not increase $\alpha 4$ levels. These results suggest that both THP and E₂ can increase expression of the GABAR $\alpha 4$ subunit, but that this effect is dependent upon the background steroid milieu as well as the degree of neuronal development. These findings demonstrate optimal conditions for steroid-induced upregulation of the $\alpha 4$ subunit in an *in vitro* system.

Keywords

pregnanolone; neurosteroid; estradiol; GABA_A receptor; $\alpha 4$; IMR-32 cells; nerve growth factor; GABA

The GABA_A receptor (GABAR) is a pentameric structure which mediates most fast inhibition in the brain, and is typically composed of 2 α , 2 β and 1 γ subunit [3]. Although the $\alpha 1$ - $\alpha 3$ subunits are most abundantly expressed in the CNS [38], the $\alpha 4$ subunit is capable of rapid plasticity. Indeed, there are numerous reports suggesting that chronic *in vivo* administration of positive GABA modulators, such as benzodiazepines and ethanol increase expression of this subunit in CNS [4,13,21]. In addition, the GABA-modulatory [22] metabolite of progesterone, THP (3 α -OH-5 α [β]-pregnan-20-one or [allo]pregnanolone), when administered to female rats at physiological doses, also increases $\alpha 4$ expression in hippocampus after 48-72 h [11,34].

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However, despite the *in vivo* evidence that chronic THP treatment can upregulate expression of the $\alpha 4$ protein, there is no direct evidence for this using *in vitro* techniques. In contrast, effects of other GABA-modulators on $\alpha 4$ expression have been established *in vitro* [7,31]. In one recent study, 7 day administration of the parent compound progesterone was seen to increase $\alpha 4$ mRNA levels in differentiated NT2-N cells [27]. However, a second study showed that 5 day application of THP reduced $\alpha 4$ mRNA levels in developing P19 cells [10]. The experimental conditions of these two studies were different than the *in vivo* protocols, in that developing cells were used, mRNA levels rather than protein levels were assessed and, in the second study, $\alpha 4$ levels were assessed beyond the 48-72 h timepoint which produces maximal effects *in vivo* [11]. In addition, cells were grown in serum-containing medium [15], where the presence of steroids such as progesterone and THP may have influenced the ability of THP to alter $\alpha 4$ expression. Thus, the present study was conducted to examine effects of 48 h THP exposure on expression of $\alpha 4$ protein under varying conditions of neuronal differentiation and background steroid levels. To this end, we used 4MA (N,N-diethyl-4-methyl-3-oxo-4-aza-5 α -androstane-17 β -carboxamide), a blocker of THP formation [36], as well as serum-free conditions to compare with effects of 48 h THP treatment on $\alpha 4$ expression in developing or differentiated IMR-32 cells. This is a well-established neuroblastoma cell line previously shown to express GABAR subunits, including $\alpha 4$ and θ , which yield functional receptors [32]. In addition, the currents recorded from these cells are responsive to neurosteroid potentiation [32], further suggesting it as an appropriate model.

Human neuroblastoma IMR-32 cells (ATCC) were maintained in EMEM (ATCC) supplemented with 2 mM L-glutamine and 10% FBS, and were incubated in humidified 5% CO₂ at 37°C. After growing for 3-4 days, cells were differentiated into neurons with one of two different protocols: i. serum deprivation or ii. 5-bromo-2-deoxyuridine (BrdU)-induced. For i, we used DMEM/F-12 (ATCC), supplemented with 0.2% BSA for 2-5 days, with or without 20 ng/ml human NGF- β [29]. For ii, we used 10 μ M BrdU (Fluka) added to DMEM/F-12 with 5% FBS for 10-11 days. All culture media contained 100 I.U./ml penicillin and 100 μ g/ml streptomycin.

In some cases 4MA (N,N-diethyl-4-methyl-3-oxo-4-aza-5 α -androstane-17 β -carboxamide, 5 α -reductase blocker, 500 nM), THP (100 nM, P8150-000, Steraloids, Newport, RI) and/or E₂ (600 pM, Steraloids) were added to the cells for 48 h (vehicle, 0.001% DMSO).

Levels of the $\alpha 4$ subunit were assessed using standard Western blot procedures as described previously [36] following isolation of cell membranes and determination of protein content. In all cases, two or three protein loading amounts were tested per sample to ensure density readings in the linear range. Cell membranes electrophoresed onto 8% or 10% Tris-Glycine or NuPage Bis-Tris gels were transferred to nitrocellulose membranes (Invitrogen). The $\alpha 4$ subunit was detected with a rabbit antibody [36] against the C-terminal end as a 67 kDa band and visualized by enhanced chemoluminescence (ECL) (Pierce). The optical density of the immunoreactive bands was analyzed using a Microtek scanner and One-DScan Gel Analysis Software. Results were normalized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control protein from the same samples (36 kDa band).

GABA-activated current was recorded using whole cell patch clamp procedures at -50 mV at room temperature (20-25°C) in a bath solution containing (in mM): NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, HEPES 10, and D-glucose 24, pH 7.4, 320 mOsm/kg H₂O. The pipette solution contained (in mM): N-methyl-D-glutamine chloride 120, Cs₄BAPTA 5, Mg-ATP 5. The ATP regeneration system, Tris phosphocreatinine (20 mM) and creatine kinase (50 U/ml), was added to minimize GABA rundown. Currents were filtered at 3 kHz and digitally sampled at 10 kHz using the pClamp 8.2 software package (Axon Instruments). Drug delivery was accomplished via a solenoid-activated rapid superfusion system positioned within 50 μ m of the cell, as

previously described [36]. Peak GABA-gated current was calculated during THP application as the percentage GABA potentiation relative to the current gated by 10 μ M GABA (EC_{20}). All chemicals were obtained from Sigma or Calbiochem unless otherwise indicated.

Data are expressed as the mean $\pm$ S.E.M. Differences between groups were assessed using standard ANOVA and Tukey (>2 groups) *post hoc* procedures. Significance was determined when $p < 0.05$.

We initially tested the effect of 48 h THP administration on GABAR $\alpha 4$ expression in undifferentiated IMR-32 cells. Cells were treated once per day with 100 nM THP in some cases following blockade of endogenous THP in the medium using the 5 α -reductase blocker 4MA (500 nM). In fact, 48 h THP treatment was effective in significantly ($P < 0.05$) increasing $\alpha 4$ expression by 2-3-fold only when basal levels of the steroid in serum-containing medium were reduced with 4MA (Fig. 1). In contrast, 48 h treatment with THP or 4MA applied alone had no effect on $\alpha 4$ expression.

Because this result suggests that the background steroid milieu is a factor in $\alpha 4$ expression, we chose the serum deprivation method with or without addition of NGF to differentiate IMR-32 cells into a neuronal phenotype. After 2-5 days in serum-free medium, IMR-32 cells grew large neuronal processes, which were increased following NGF administration (20 ng/ml), as previously described [29].

We initially verified that GABA application generated bicuculline-sensitive currents following NGF treatment of IMR-32 cells differentiated in serum-free medium (Fig. 2A), as has been reported in undifferentiated IMR-32 cells [32]. Further, 100 nM THP application significantly ($P < 0.05$) potentiated this GABA(EC_{20})-gated current by 102 ± 12 % in NGF-treated differentiated IMR-32 cells ($n=13$), but was without effect in the absence of GABA application (Fig. 2B), suggesting that this steroid is a potent positive GABA-modulator as described in other systems [22,32].

Under conditions of serum deprivation, combined administration of THP plus E_2 significantly increased $\alpha 4$ expression by 2-3-fold (Fig. 3E,F), while neither steroid alone was effective in this regard. However, following treatment with NGF, all steroid treatment regimens produced significant increases in $\alpha 4$ expression: THP; E_2 ; and THP plus E_2 ($P < 0.05$). In contrast, THP was ineffective in upregulating $\alpha 4$ expression following neuronal differentiation in serum-containing medium (Fig. 3C,D). In addition, neuronal differentiation by serum deprivation alone (Fig. 3A, B) did not alter $\alpha 4$ expression compared to the undifferentiated state of the cells. These data suggest that neuronal differentiation by serum deprivation plus NGF administration is the optimal condition for steroid-induced increases in $\alpha 4$ expression in IMR-32 cells by THP as well as by E_2 .

The results from this study suggest that both the background steroid milieu as well as the developmental stage of the IMR-32 cell influence the ability of THP to increase expression of the $\alpha 4$ subunit of the GABAR. In both differentiated and undifferentiated IMR-32 cells, THP produced significant effects on $\alpha 4$ expression only with low background steroid levels. These results are consistent with findings from both in vitro and behavioral studies showing that THP exerts more significant effects when endogenous THP levels are lowest across the estrous cycle [6]. Unpublished results from this laboratory also demonstrate that THP administration to female mice does not alter $\alpha 4$ expression when administered during the nocturnal surge in CNS THP levels. These findings imply that the critical factor regulating $\alpha 4$ expression is not the absolute level of steroid, but rather the change in steroid level which triggers $\alpha 4$ expression, as has been suggested by other groups [18].

In differentiated neuroblastoma cells grown in serum-free medium to lower the ambient steroid levels, THP produced significant effects on $\alpha 4$ expression only following NGF treatment. The increased development of neuronal processes produced by NGF [29] may be responsible for this effect, as $\alpha 4$ expression in native receptors has been localized to dendritic processes rather than the perisomatic region [25]. Alternatively, expression of factors involved in transcription or trafficking may also play a role. In fact, NGF is known to increase several cell signaling cascades [14], including Map Kinase, PI 3-kinase and phospholipase C- γ , as well as a diverse array of G-proteins, which are known to regulate receptor trafficking in neurons, while early growth factor (Egr) is a key element in activating the $\alpha 4$ promoter [30]. However, our data as well as other studies [27] have not observed steroid-induced upregulation of the $\alpha 4$ subunit after 48 h treatment of differentiated neurons, suggesting that this event requires both differentiation as well as a serum-free environment.

Interestingly, 48 h E₂ exposure was also effective in increasing $\alpha 4$ expression in NGF-treated cells. It has been shown that long-term exposure to NGF enhances E₂ binding in neuronal differentiating PC12 cells. Reciprocally, E₂ up-regulates TrkA mRNA and transiently down-regulates p75 mRNA [23], which are NGF receptors, suggesting that E₂ may increase the efficiency of NGF binding in IMR-32 which express TrkA but not p75 [12]. Another recent study has shown that E₂ can increase $\alpha 4$ expression in NT2-N cells after a 7-day exposure [27]. The difference in timecourse may be due to the fact that serum-free medium was used in the present study to reduce ambient steroid levels, which would be expected to increase steroid sensitivity. In addition, differences in characteristics of cell lines and maturity of neurons [24,32] might also play a role.

Our findings also suggest that THP and E₂ produce additive effects, consistent with the idea that these steroids act via distinct mechanisms. THP is a positive modulator of the GABAR [22], while E₂ has no direct effect at GABAR [22], but can initiate a variety of cell signaling mechanisms known to affect $\alpha 4$ transcription and receptor trafficking [16], such as PKC [28], which can also increase activation of the $\alpha 4$ promoter [30]. Our earlier in vivo work also suggested that E₂ could enhance the ability of progesterone, the parent compound of THP, to increase $\alpha 4$ expression [34]. The recently characterized $\alpha 4$ promoter [20] has multiple start sites and lacks a conventional TATA box, suggesting that it is regulated by a variety of conditions, consistent with the findings from the present study.

The effect of E₂ may also be related to indirect effects on neuronal development. Recent studies suggest that E₂ can increase brain-derived neurotrophic factor in the CNS [33,39], leading to dendritic plasticity and neuronal development [33]. In addition, it has been reported that 48 h E₂ exposure prolongs and enhances Ca transients induced by GABAR activation or voltage-gated channels in developing neurons [26]. Calcium influx has been identified as a critical factor in GABAR regulation in response to prolonged GABA exposure [19], and may contribute to the observed effect of E₂ in the present study.

The results from the present study are consistent with our previous in vivo findings, which demonstrated that 48 h exposure of female rats to THP increases expression of the $\alpha 4$ subunit in CA1 hippocampus [11,34]. The timecourse of steroid exposure required for $\alpha 4$ expression in fact was in the narrow range of 48-72 h, where longer exposures returned $\alpha 4$ expression back to control levels [11], although withdrawal from the steroid was also effective in increasing $\alpha 4$ expression [36]. In fact, one study which used a longer exposure time reported a decrease in $\alpha 4$ mRNA expression, although this reversed 24 h after withdrawal [10]. Other studies have reported increases in $\alpha 4$ expression in other CNS areas, such as the periaqueductal grey, after steroid withdrawal [9]. Study of the withdrawal effects of the steroid require a 21 day exposure period [36] which is not possible with the current model, and awaits further study.

It is entirely possible that different mechanisms mediate the effects of chronic steroid administration and steroid withdrawal on $\alpha 4$ expression, however.

$\alpha 4$ -containing GABAR have unique kinetic properties compared to the more abundantly expressed $\alpha 1$ -, $\alpha 2$ -, and $\alpha 3$ -containing GABAR [8,17]. They deactivate more quickly [35], with a faster τ -fast, and have a lower open probability as well as a significantly shorter mean open time [1]. These characteristics would tend to decrease the total charge transfer, and reduce the inhibition produced by current gated through these receptors. Indeed, increases in $\alpha 4$ expression are seen in models of temporal lobe epilepsy [2], as well as during the withdrawal hyperexcitability and increased anxiety that follows chronic administration of GABA-modulatory compounds [4,7,13,21,36]. In fact, down-regulation of $\alpha 4$ expression with in vivo antisense treatment prevents increases in seizure susceptibility following neurosteroid withdrawal [36], thus providing evidence that these faster deactivating receptors may mediate hyperexcitability states. Thus, regulation of the expression of the $\alpha 4$ subunit has significant consequences for the behavioral state of the animal.

In addition, $\alpha 4$ -containing GABARs are insensitive to modulation by benzodiazepines [38]. These characteristics suggest that alterations in $\alpha 4$ expression may be relevant for premenstrual dysphoric disorder, when increased anxiety [5] and decreased benzodiazepine responsiveness [37] have been reported shortly after the onset of the luteal phase, following increases in endogenous, circulating levels of THP. The results from this study may suggest conditions which underlie changes in $\alpha 4$ expression relevant for changes in mood and behavior across the menstrual cycle.

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References

- [1]. Akk G, Bracamontes J, Steinbach JH. Activation of GABA_A receptors containing the [alpha]4 subunit by GABA and pentobarbital. *J. Physiol* 2004;556:387–399. [PubMed: 14966300]
- [2]. Brooks-Kayal AR, Shumate MD, Jin H, Rikhter TY, Coulter DA. Selective changes in single cell GABA_A receptor subunit expression and function in temporal lobe epilepsy. *Nat. Med* 1998;4:1166–1172. [PubMed: 9771750]
- [3]. Chang Y, Wang R, Barot S, Weiss DS. Stoichiometry of a recombinant GABA_A receptor. *J. Neurosci* 1996;16:5415–5424. [PubMed: 8757254]
- [4]. Devaud LL, Fritschy JM, Sieghart W, Morrow AL. Bidirectional alterations of GABA_A receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. *J. Neurochem* 1997;69:126–130. [PubMed: 9202302]
- [5]. Endicott J, Amsterdam J, Eriksson E, Frank E, Freeman E, Hirschfeld R, Ling F, Parry B, Pearlstein T, Rosenbaum J, Rubinow D, Schmidt P, Severino S, Steiner M, Stewart DE, Thys-Jacobs S. Is premenstrual dysphoric disorder a distinct clinical entity? *J. Womens Health Gend. Based Med* 1999;8:663–679. [PubMed: 10839653]
- [6]. Finn DA, Gee KW. The influence of estrus cycle on neurosteroid potency at the gamma-aminobutyric acidA receptor complex. *J. Pharmacol. Exp. Ther* 1993;265:1374–1379. [PubMed: 8389864]
- [7]. Follesa P, Cagetti E, Mancuso L, Biggio F, Manca A, Maciocco E, Massa F, Speranza Desole M, Carta M, Busonero F. Increase in expression of the GABA_A receptor [alpha]4 subunit gene induced by withdrawal of, but not by long-term treatment with, benzodiazepine full or partial agonists. *Mol. Brain Res* 2001;92:138–148. [PubMed: 11483250]
- [8]. Gingrich KJ, Roberts WA, Kass RS. Dependence of the GABA_A receptor gating kinetics on the [alpha]-subunit isoform: implications for structure-function relations and synaptic transmission. *J. Physiol* 1995;489:529–543. [PubMed: 8847645]

- [9]. Griffiths J, Lovick T. Withdrawal from progesterone increases expression of [alpha]4, [beta]1, and [delta] GABA_A receptor subunits in neurons in the periaqueductal gray matter in female Wistar rats. *J. Comp. Neurol* 2005;486:89–97. [PubMed: 15834956]
- [10]. Grobin AC, Morrow AL. 3[alpha]-hydroxy-5[alpha]-pregnan-20-one exposure reduces GABA_A receptor [alpha]4 subunit mRNA levels. *Eur. J. Pharmacol* 2000;409:R1–2. [PubMed: 11104837]
- [11]. Gulinello M, Gong QH, Li X, Smith SS. Short-term exposure to a neuroactive steroid increases [alpha]4 GABA_A receptor subunit levels in association with increased anxiety in the female rat. *Brain Res* 2001;910:55–66. [PubMed: 11489254]
- [12]. Hartman DS, Hertel C. Nerve growth factor-induced differentiation in neuroblastoma cells expressing TrkA but lacking p75NGFR. *J. Neurochem* 1994;63:1261–1270. [PubMed: 7523588]
- [13]. Holt RA, Bateson AN, Martin IL. Chronic treatment with diazepam or abecarnil differently affects the expression of GABA_A receptor subunit mRNAs in the rat cortex. *Neuropharmacol* 1996;35:1457–1463.
- [14]. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem* 2003;72:609–642. [PubMed: 12676795]
- [15]. Jung-Testas I, Baulieu EE. Effects of sex steroids and antihormones on growth, adhesiveness and receptors of L-929 cells cultured in serum-containing and serum-free media. *Exp. Cell Res* 1979;119:75–85. [PubMed: 216565]
- [16]. Kittler JT, Moss SJ. Modulation of GABA_A receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. *Curr. Opin. Neurobiol* 2003;13:341–347. [PubMed: 12850219]
- [17]. Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB, Twyman RE. Activation and deactivation rates of recombinant GABA_A receptor channels are dependent on alpha-subunit isoform. *Biophys. J* 1997;73:1–9.
- [18]. Lovick TA. Plasticity of GABA-A receptor subunit expression during the oestrous cycle of the rat: implications for premenstrual syndrome in women. *Exp. Physiol* 2006;91:655–660. [PubMed: 16740643]
- [19]. Lyons HR, Land MB, Gibbs TT, Farb DH. Distinct signal transduction pathways for GABA-induced GABA_A receptor down-regulation and uncoupling in neuronal culture: a role for voltage-gated calcium channels. *J. Neurochem* 2001;78:1114–1126. [PubMed: 11553685]
- [20]. Ma L, Song L, Radoi GE, Harrison NL. Transcriptional regulation of the mouse gene encoding the [alpha]4 subunit of the GABA_A receptor. *J. Biol. Chem* 2004;279:40451–40461. [PubMed: 15265862]
- [21]. Mahmoudi M, Kang M-H, Tillakaratne N, Tobin AJ, Olsen RW. Chronic intermittent ethanol treatment in rats increases GABA_A receptor [alpha]4 subunit expression: possible relevance to alcohol dependence. *J. Neurochem* 1997;68:2485–2492. [PubMed: 9166743]
- [22]. Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* 1986;232:1004–1007. [PubMed: 2422758]
- [23]. Miranda RC, Sohrabji F, Toran-Allerand D. Interactions of estrogen with the neurotrophins and their receptors during neural development. *Horm. Behav* 1994;28:367–375. [PubMed: 7729805]
- [24]. Neelands TR, Zhang J, Macdonald RL. GABA_A receptors expressed in undifferentiated human teratocarcinoma NT2 cells differ from those expressed by differentiated NT2-N cells. *J. Neurosci* 1999;19:7057–7065. [PubMed: 10436060]
- [25]. Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, Houser CR. GABA(A) receptor changes in delta subunit-deficient mice: altered expression of alpha4 and gamma2 subunits in the forebrain. *J Comp Neurol* 2002;446:179–197. [PubMed: 11932935]
- [26]. Perrot-Sinal TS, Davis AM, Gregerson KA, Kao JPY, McCarthy MM. Estradiol enhances excitatory [gamma]-aminobutyric acid-mediated calcium signaling in neonatal hypothalamic neurons. *Endocrinol* 2001;142:2238–2243.
- [27]. Pierson RC, Lyons AM, Greenfield LJ Jr. Gonadal steroids regulate GABA_A receptor subunit mRNA expression in NT2-N neurons. *Brain Res. Mol. Brain Res* 2005;138:105–115. [PubMed: 16000229]

- [28]. Qiu J, Bosch MA, Grandy DK, Tobias SC, Scanlan TS, Ronnekleiv OK, Kelly MJ. Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. *J. Neurosci* 2003;23:9529–9540. [PubMed: 14573532]
- [29]. Reynolds CP, Perez-Polo JR. Induction of neurite outgrowth in the IMR-32 human neuroblastoma cell line by nerve growth factor. *J. Neurosci. Res* 1981;6:319–325. [PubMed: 6271984]
- [30]. Roberts DS, Raol YH, Bandyopadhyay S, Lund IV, Budreck EC, Passini MA, Wolfe JH, Brooks-Kayal AR, Russek SJ. Egr3 stimulation of GABRA4 promoter activity as a mechanism for seizure-induced up-regulation of GABA_A receptor [alpha]4 subunit expression. *Proc. Natl. Acad. Sci. USA* 2005;102:11894–11899. [PubMed: 16091474]
- [31]. Sanna E, Mostallino MC, Busonero F, Talani G, Tranquilli S, Mameli M, Spiga S, Follesa P, Biggio G. Changes in GABA_A receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. *J. Neurosci* 2003;23:11711–11724. [PubMed: 14684873]
- [32]. Sapp DW, Yeh HH. Heterogeneity of GABA_A receptor-mediated responses in the human IMR-32 neuroblastoma cell line. *J. Neurosci. Res* 2000;60:504–510. [PubMed: 10797553]
- [33]. Scharfman HE, MacLusky NJ. Similarities between actions of estrogen and BDNF in the hippocampus: coincidence or clue? *Trends Neurosci* 2005;28:79–85. [PubMed: 15667930]
- [34]. Shen H, Gong QH, Yuan M, Smith SS. Short-term steroid treatment increases [delta] GABA_A receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects. *Neuropharmacol* 2005;49:573–586.
- [35]. Smith SS, Gong QH. Neurosteroid administration and withdrawal alter GABA_A receptor kinetics in CA1 hippocampus of female rats. *J. Physiol* 2005;564:421–436. [PubMed: 15705652]
- [36]. Smith SS, Gong QH, Hsu FC, Markowitz RS, French-Mullen JM, Li X. GABA_A receptor [alpha]4 subunit suppression prevents withdrawal properties of an endogenous steroid. *Nature* 1998;392:926–930. [PubMed: 9582073]
- [37]. Sundstrom I, Nyberg S, Backstrom T. Patients with premenstrual syndrome have reduced sensitivity to midazolam compared to control subjects. *Neuropsychopharmacol* 1997;17:370–381.
- [38]. Wisden W, Herb A, Wieland H, Keinänen K, Luddens H, Seeburg PH. Cloning, pharmacological characteristics and expression pattern of the rat GABA_A receptor [alpha]4 subunit. *FEBS Lett* 1991;289:227–230. [PubMed: 1655526]
- [39]. Zhou J, Zhang H, Cohen RS, Pandey SC. Effects of estrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures. *Neuroendocrinol* 2005;81:294–310.

Fig 1. 48 h THP administration alters expression of the $\alpha 4$ subunit of the GABA_A receptor in undifferentiated IMR-32 cells. A, Representative Western blot of $\alpha 4$ and GAPDH immunoreactivity. Con, control. B, Statistical analysis of the data is represented as the integrated optical density of the immunoreactive bands relative to the GAPDH level and is expressed as a ratio of the control condition (mean $\pm$ S.E.M.). (ANOVA, * $p < 0.05$ vs. Con, $n = 8$).

Fig 2. NGF-differentiated IMR-32 cells express functional GABA_A receptors which are steroid-sensitive. A, GABA-gated whole-cell current was completely blocked by bicuculline. Representative of results from 6 cells. Horizontal bars, drug application. B, 100 nM THP potentiated 10 μ M GABA-induced current by 102 % (mean), but did not elicit a current without GABA application. Representative of results from 13 cells.

Fig 3.

48 h steroid treatment increases $\alpha 4$ expression in differentiated IMR-32 cells: An effect dependent upon nerve growth factor. Representative Western blot (A) and averaged data (B) of $\alpha 4$ and GAPDH immunoreactivity in differentiated (Diff) and undifferentiated (Undiff) IMR32 cells (10 μ g loading protein). There are no significant differences in $\alpha 4$ expression between these two stages of cells (Mean $\pm$ SEM, $p > 0.05$, $n = 4$). C, BrdU-differentiated IMR-32 cells in serum-containing medium. THP did not increase $\alpha 4$ expression. D, Averaged data, $P > 0.05$, $n = 4$. E, In contrast, $E_2 +$ THP treatment increased $\alpha 4$ expression in differentiated IMR-32 cells cultured without NGF (left), while 48 h treatment with THP, E_2 or $E_2 +$ THP all increased $\alpha 4$ expression in cells differentiated with 100 nM NGF (right). F, Averaged optical

densities (mean $\pm$ S.E.M), normalized to GAPDH level and expressed relative to control. (ANOVA, * $p < 0.05$ vs. control. $n = 6$; ** $p < 0.05$ vs. NGF. $n = 5$).