Plasma glucocorticoids differentially modulate phasic and tonic GABA inhibition during early postnatal development in rat spinal lamina II

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HIGHLIGHTS

• Plasma CORT regulates phasic but not tonic GABA spinal inhibition.
• Hypercortisolemic rats display enhanced phasic GABAA inhibition during development.
• Rat with high plasma CORT level exhibited reduced mechanical pain sensitivity.
• Extrasynaptic GABAA current increases during the first postnatal weeks.

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ABSTRACT

Noxious processing is tuned by GABA receptor-mediated inhibition in the spinal cord dorsal horn that undergoes postnatal maturation in rodents. These GABAergic inhibitory postsynaptic currents (IPSCs) are modulated by 3α5α-reduced steroids during early postnatal development in spinal cord lamina II. Thus an enhanced phasic inhibition is present in neonates and decreases over time. GABA can also activate extrasynaptic receptors, giving rise to tonic inhibition. In this study, we characterized the contribution of plasma corticosterone (CORT) to postnatal maturation of spinal phasic and, for the first time, tonic GABAergic inhibitions. We used Fisher and Lewis rat strains displaying respectively high and low hypothalamic-pituitary-adrenal axis reactivity, compared to control Sprague-Dawley rats. Measured plasma CORT levels were significantly higher in Fisher rats, which also displayed significantly higher mechanical nociceptive thresholds, supporting the hypothesis of an antinociceptive action of CORT. Recorded GABA IPSCs shortened during maturation in all strains while remaining larger in Fisher rats. Blocking the 3α-reduction of steroids in Fisher rats produced a further decrease of IPSC deactivation time constant. In contrast, GABA tonic inhibition progressively increased during maturation, without any difference among strains. In conclusion, we show that both phasic and tonic GABAergic inhibitions undergo postnatal maturation in lamina II. Moreover spinal production of 3α5α-reduced steroids that presumably derive from plasma CORT is correlated to spinal GABA phasic (but not tonic) inhibition and to mechanical nociceptive thresholds.

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In rodents, inhibition in the dorsal spinal cord plays a major role in sensory information processing. In spinal superficial laminae, fast inhibitory synaptic transmission is mediated by both glycine and GABA A receptors (GABA A R) [1]. GABA A phasic inhibition mediated by synaptic receptors is high at birth and then progressively decreases during early postnatal development [2]. Besides, it has been shown that enhanced inhibitory transmission prevents or alleviates hyperalgesia and allodynia [3,4]. Apart from phasic inhibition, a tonic GABAergic inhibition mediated by extrasynaptic GABA A R has also been reported [5]. Both types of GABA inhibitions have different spatiotemporal resolutions. Due to its properties, the tonic inhibition acts as a major filter, which reduces neuronal network excitability [6]. However, to our knowledge little is known regarding the early postnatal

Abbreviations: 3α5αNS, 3α5α-reduced neurosteroids; ACSF, artificial cerebrospinal fluid; BIC, bicuculline; CORT, corticosterone; FIN, finasteride; GABA A R, GABA A receptor; FIS, Fischer; GABA, γ-aminobutyric acid; GBZ, gabazine; GC, glucocorticoids; HPA, hypothalamic-pituitary-adrenal axis; IPSC, inhibitory postsynaptic current; LEW, Lewis; PX, X postnatal day; RIA, radioimmunoassay; SD, Sprague-Dawley; SHRP, stress hyposensitive period.

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maturation of the tonic component of GABA_A inhibition in dorsal spinal cord.

Previous studies have shown that steroids play a key role in shaping GABA_A inhibitory postsynaptic currents (IPSCs) in superficial laminae [2,7,8]. 3α5α-reduced steroids are potent positive allosteric modulators of GABA_A,R, which increases channel open probability and duration [9]. The consequence is a slowing down of the deactivation time constant leading to an increase of apparent affinity [10,11]. Moreover, it has been shown that spinal production of 3α5α-reduced steroids is developmentally regulated. The production is high at birth, decreases thereafter and GABA IPSC modulation by 3α5α-reduced steroids is absent after P21 [2,8]. However in adult rats, inflammatory pain is able to up-regulate spinal production of 3α5α-steroids [12].

Synaptic synthesis of 3α5α-steroids can be fully achieved from cholesterol. However, plasma steroids produced by adrenals glands or gonads can freely cross the blood brain barrier, and can be spinally reduced to modulate GABA_A inhibition as demonstrated for progesterone [13].

Glucocorticoids (GC) production by the adrenal cortex under the control of the hypothamic-pituitary-adrenal (HPA) axis is developmentally regulated [14]. Particularly in the stress hyporesponsive period (SRHP) during the early postnatal stages, HPA axis is silent in rodents [15]. In the current work, we used three rat strains displaying different HPA axis reactivity: inbred Lewis (LEW) and Fischer 344 (FIS) rats showing a genetically depressed or enhanced release of CORT respectively [16,17] and Sprague-Dawley (SD) rats serving as controls. Interestingly, FIS have been shown to recover faster from inflammatory pain [18], thus appearing to be more protected from pathological pain. Although high levels of GC may profoundly alter pain perception and give rise to allodynia [19,20], little is known regarding a direct modulation of the spinal neuronal network by plasma GC, despite the fact THDOC (tetrahydrodeoxy-corticosterone), a reduced GC, is a well known positive allosteric modulator of GABA_A,R [11].

The purpose of our work was to study the impact of plasma GC on tonic and phasic GABA_A inhibition in lamina II spinal neurons during the first four postnatal weeks. Therefore we combined in vitro patch-clamp recordings with radioimmunoassay measurements and behavioral tests in rats displaying differential plasma CORT levels.

1. Experimental procedures

1.1. Animals

Experiments, conducted according to European regulations and approved by the French Departmental Veterinary Service and the Animal Care and Use Committee of Luxembourg University, were performed with 5–30 days old rats bred in laboratory animal house. Rats were housed in 7–19 h light/dark cycle at 22 °C with ad libitum access to food and water.

1.2. Spinal cord slice preparation and solutions

Transverse spinal cord slices were prepared as described previously [2]. Rats were anesthetized with ketamine (Imalgene 1000, Merial, France, 75 mg/kg, i.p.) and decapitated. Spinal cord was removed by hydraulic extrusion and immersed in 4°C sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM): 248 sucrose, 11 glucose, 26 NaHCO_3, 2 KCl, 1.25 K_H2PO_4, 2 CaCl_2, 1.3 MgSO_4 bubbled with 95% O₂–5% CO₂, pH 7.35. After meninges removal, lumbar segment was cut transversally (Leica 1200vs). Slices (600 μm) were stored at room temperature in ACSF containing 126 mM of NaCl instead of sucrose, then transferred in recording chamber perfused with oxygenated ACSF.

1.3. Electrophysiological recordings and data acquisition

Whole-cell voltage clamp recordings were obtained with a Multiclamp 700B amplifier (Axon Instruments). Borosilicate glass electrodes (OD: 1.2, ID: 0.69; Harvard Apparatus, UK) were pulled using a P2000 puller (Sutter Instruments). Pipettes (3–5 MΩ) were filled with intracellular solution containing (in mM): 80 CsSO_4, 2 MgCl_2, 8 KC1, 10 HEPES, 2 MgATP, 0.2 NaGTP, pH 7.3. Series resistances and capacitances were electronically compensated. Recordings performed at room temperature were filtered at 2 kHz and digitized at 10 kHz with pClamp 8.0 (Molecular Devices).

1.4. Data analysis

Spontaneous synaptic currents were detected and analyzed using WinEDR and WinWCP (Strathclyde University, UK). Deactivation time constant was fitted using monoeponential function. Mean deactivation time constant (τ_d) was calculated by averaging at least 20 events from 5 to 17 neurons. As previously characterized [2], IPSCs with τ_d > 15 ms, sensitive to gabazine and insensitive to strychnine corresponded to GABA_A IPSCs.

Extrasynaptic GABA_A current was measured using a Gaussian function applied on an all-point histogram (p-Clamp 8) of a 2 min recording and IPSCs were excluded using cursors [21]. To avoid cell-to-cell variations, extrasynaptic current was normalized to membrane capacitance and expressed as current density (pA/μF).

1.5. Drug application

Bicuculline methiodide (BIC) or gabazine (GBZ) (10 μM each, Sigma–Aldrich) were bath applied and no difference was observed between them. GABA_A IPSCs were isolated using strychnine (1 μM, Sigma–Aldrich). Slices were incubated in finasteride (1,5α-androsten-4-aza-3-one-17β-(N-tert-butylcarboxamid), 50 μM, Steraloids) at least 3 h before recording as previously described [8].

1.6. Blood sampling and radioimmunoassay

Blood samples were taken from P8, P14, P21 and P28 rats between 07:00 and 10:00 am. 500 μl of blood was collected in EDTA-coated capillary tubes (CB300, Microvette). Blood samples were centrifuged for 15 min at 3000 rpm and plasma was collected and stored at –20 °C. Quantification of corticosterone, we made with RIA kit (Corticosterone DA 125L, MP Biomedical, France) following manufacturer’s instructions.

1.7. Mechanical nociception

Calibrated forceps (Bioseb, France) were used to measure mechanical nociceptive thresholds of P21–28 rats, as previously described [22]. Briefly, gradually increasing pressure (in g) was applied to the hind paw until aversive paw withdrawal reflexes were displayed. Right and left hind paws were tested three times and averages were measured.

1.8. Statistical analysis

Analyses were performed with STATISTICA (StatSoft). Multifactorial ANOVA followed by Tukey–Kramer post hoc test was used for multiple comparisons. Simple comparisons were done with unpaired Student’s t-test. Significance levels are: p < 0.05 (*);
p < 0.01 (**); p < 0.001 (***) Exact p-values are given in text for non-significant (N.S.) comparisons.

2. Results

2.1. Developmental changes in plasma CORT levels and relationship with pain sensitivity in mature rat

Plasma CORT levels turned out to be very low at P8 (in ng/ml: FIS: 7.39 ± 1.31, SD: 18.05 ± 4.92, LEW: 9.87 ± 3.23) and P14 (in ng/ml: FIS: 21.88 ± 3.55, SD: 40.17 ± 6.70, LEW: 35.04 ± 9.88, n = 6 rats per time point and strain, Fig. 1A). No significant differences could be found between the strains. CORT levels progressively increased in all strains after P14, and were significantly higher in FIS at P21 and P28 (ANOVA [CORT]strain × time, F6,59 = 21.61. p < 0.001) compared to SD (p < 0.001) and LEW (p < 0.001). Plasma CORT levels of SD and LEW rats were not significantly different at P21 (p = 0.998) nor at P28 (p = 0.999).

We measured mechanical nociceptive thresholds of P21–28 FIS, SD and LEW rats to evaluate if the measured plasma CORT levels could influence mechanical nociceptive sensitivity (Fig. 1B). No difference was seen between SD and LEW rats (ANOVA, F2,24 = 7.514, p = 0.047) that exhibited pressure thresholds of T38.53 ± 5.85 g (n = 12) and 125.89 ± 11.24 g (n = 6), respectively. In contrast, FIS rats displayed a significantly higher mechanical threshold compared to LEW (p < 0.01) and SD (p < 0.05), with a mean pressure threshold of 167.94 ± 7.01 g (n = 9).

2.2. Reduced GC metabolites control GABA(A) IPSC kinetics

In order to assess the impact of plasma CORT concentrations on GABA(A) synaptic inhibition, we performed whole-cell patch clamp recordings of lamina II neurons from P5 to P30 FIS, LEW and SD rats (Fig. 2A). Peak amplitude or frequency of GABA(A) IPSC occurrence showed no significant difference between P5 and P30 in the three strains. However, a reduction of the deactivation time constant (τD) was observed (ANOVA [τDstrain × time, F8,113 = 4.443, p < 0.001, n = 6 per group and per time point, Fig. 2B), between P5 and P30 for FIS (p < 0.001), SD (p < 0.001) and LEW (p < 0.05). Interestingly, the mean τD was greater in FIS at each time point compared to LEW and SD. Focusing on P5–10, we observed that τD was clearly slower in FIS (48.74 ± 1.43 ms, n = 14) compared to SD (32.90 ± 1.62 ms, n = 18, p < 0.001) and LEW rats (25.27 ± 2.36 ms, n = 7, p < 0.001) that exhibited the fastest decaying kinetics. After at P21 mean τD stabilized but FIS rats still showed slower decays of GABA(A) IPSCs (27.30 ± 1.23 ms, n = 13) compared to LEW (20.64 ± 0.67 ms, n = 19, p < 0.001) and SD (21.20 ± 1.24 ms, n = 10, p < 0.001), both of which exhibited similar mean τD (p = 0.885).

Next, we incubated slices from P8 and P21 FIS rats with the 5α-reductase inhibitor finasteride (FIN, 50 μM) to verify if the larger τD is a consequence of elevated levels of GC locally reduced in 3α5α. As shown in Fig. 2B, after incubation of at least 3 h, τD were significantly smaller at P8 (Control: 48.81 ± 1.54 ms (n = 13); FIN: 27.89 ± 1.14 ms (n = 13)) and at P21 (control: 27.30 ± 1.23 ms (n = 13); FIN: 20.49 ± 0.91 ms (n = 8); p < 0.001). Note that in FIN, at P21, the mean τD after FIN incubation reached a value that was similar to that of SD or LEW (ANOVA, F2,34 = 0.346, p = 0.225).

2.3. Developmental regulation of tonic GABAergic inhibition

In all recorded neurons and strains at P8, P14 and P21, we found that application of the GABA(A)R antagonist GBZ (10 μM) produced a decrease in the mean baseline current (Fig. 2C) that suggests that extrasynaptic GABA(A)Rs are tonically activated. The GBZ-sensitive
current density increased significantly over early postnatal maturation. It almost tripled in the three strains between P8 and P21 (in pA/pF; FIS: from 0.16 ± 0.04 (n = 7) to 0.57 ± 0.15 (n = 6); SD: from 0.21 ± 0.05 (n = 10) to 0.62 ± 0.20 (n = 8); LEW: from 0.21 ± 0.07 (n = 6) to 0.62 ± 0.09 (n = 6); ANOVA, F_{2,50} = 8.637, p < 0.001). No difference could be found between strains at any time point (ANOVA, F_{2,50} = 0.103, p = 0.902).

Finally, we investigated the impact of the synaptic GABA current frequency on GABAergic extrasynaptic current density. Although the frequency of GABA IPSCs did not vary during maturation (ANOVA [frequency]_strain × time, F_{3,72} = 1.514, p = 0.227), we expressed GABAergic extrasynaptic current as a function of GABA IPSCs frequency and linear regression analysis showed no significant correlation at P8 (R^2 = 0.04, p = 0.531) or at P21 (R^2 = 0.01, p = 0.801).

3. Discussion

The data presented here suggest that plasma GCs impact mechanical nociceptivity sensitivity via a modulation of spinal inhibition. This effect seems to be mediated, at least in the spinal cord, by the fine-tuning of synaptic rather than extrasynaptic GABAAR activity.

3.1. Plasma CORT concentration and IPSC maturation in lamina II

GABA is a well-known inhibitory neurotransmitter of spinal cord lamina II. It takes part to the modulation and integration of nociceptive information before its transmission to supraspinal structures [4]. In our study, we performed in vitro patch clamp recordings from spinal cord slices of rats ranging from neonatal to mature stages to describe maturation of spinal GABAergic inhibition in animals expressing different levels of plasma CORT.

It has been widely demonstrated in CNS that postnatal maturation of GABAergic inhibition is regulated by 3x5α-reduced neurosteroids (3x5αNS). For instance, Keller et al. [2,8] have shown that a sustained 3x5αNS production in spinal cord lamina II is sufficient to prolong GABA miniature IPSCs in early developmental stages. These investigators, working with SD strain, have also shown that spinal 3x5αNS production decreases during the first four postnatal weeks and then disappeared. Our results are consistent with these reports since we observed such an acceleration of GABAAR IPSC kinetics in SD, as well as in FIS and LEW strains.

Nevertheless, another explanation could be a change in GABAAR subunits composition. Okada et al. (2000) demonstrated in thalamus that the developmental acceleration of GABA IPSCs kinetics is linked to α1 subunit expression [23]. However, this subunit is not present in lamina II, thus our recorded changes are more likely due to a steroid modulation [24].

Mature FIS rats, which have high plasma GC levels, displayed longer synaptic inputs than LEW and SD. Furthermore in FIS, FIN incubation reduced synaptic input duration at P8, corroborating previous results, but more interestingly, also after P21 (Fig. 2B), at a time point where Keller et al. [2,8] did not detect any 3x5αNS production in SD rats. Our findings highlight the production of 3x5αNS during late spinal maturation in FIS, whereas neurosteroidogenesis from cholesterol incorporation in mitochondria is no longer detected in other strains [12]. We then assume that in mature FIS, 3x5αNS are produced in spinal cord from plasma GC crossing the blood brain barrier. Indeed, De Nicola et al. showed that spinal CORT level follows its peripheral variations [25]. Moreover Mensah-Nyagan et al. have demonstrated that enzymes implicated in this reduction pathway are expressed and functional.

Therefore spinal reduction of CORT in THDOC would compensate for the decreasing tonus of endogenous 3x5αNS produced from cholesterol incorporation in mitochondria. The THDOC so produced, a positive allosteric modulator of GABAAR receptor, would then maintain or increase spinal synaptical inhibition. Nevertheless, we cannot exclude that another 3αx5αNS (allopregnanolone) known to prolong GABA IPSC [12] could also take part to this modulation.

In LEW and SD, GC production is lower and plasma precursors of 3x5αNS reaching the dorsal horn may not be sufficient to affect spinal GABAergic inhibition, particularly because our experiments have been performed at the nadir of CORT production (09:00 a.m.). We can however not exclude that plasma CORT may exert such an influence in SD and LEW when its production reaches maximal levels, e.g. before waking-up (07:00 p.m.) or under stress conditions.

Our experiments focused only on non-genomic allosteric actions of reduced GC. We can hence not exclude a genomic action of GC mediated through their cytosolic glucocorticoid and mineralocorticoid receptors that are present in the dorsal spinal cord [27,28]. Nevertheless, our results strongly suggest that allosteric GABAAR modulation by reduced-steroids is sufficient to explain CORT spinal action on the integration of nociceptive information.

3.2. Inhibitory GABAergic extrasynaptic current maturation in lamina II

To our knowledge, the present paper provides the first characterization of the maturation of GABAAR current in lamina II neurons. Indeed, we have recorded tonic GABAergic inhibition as early as P8 and this inhibition increased with time (Fig. 2C). The current density that we measured at P21 is comparable to the one observed in 3–6 month-old mice by Bonin et al. [20]. These findings tend to indicate that the extrasynaptic GABAergic current has reached its maximum after P21. The observed increase in tonic inhibition can be explained by several mechanisms: changes in diffusion properties of the extracellular space, progressive increases in extrasynaptic GABAAR density, increased activity of GABAergic interneurons, as well as by a change in the phosphorylation of GABAAR which has been shown to affect GABAAR affinity [29]. Another possibility would be a change in GABAAR subunit composition, however, to our knowledge no data are available regarding developmental changes in spinal α4, α5, α6 or δ subunit expression. We have also excluded an impact of an increased synaptic GABA release frequency. Indeed, previous studies have shown that during high IPSC frequency GABA can reach millimolar concentrations in the synaptic cleft, leading to a saturation of neuronal and glial GABA transporters and giving rise to spillover in the extraspinal space [21,30]. In the present study, we did not observe any correlation between GABA IPSC frequency and maturation. Thus, the increase of extrasynaptic current amplitude that we measured during postnatal maturation is not likely to result from increased spillover.

3.3. Functional implication of GC action on spinal inhibition

Tonic GABAergic inhibition affects overall neuronal network excitability, whereas fast inhibition specifically acts on temporally correlated synaptic inputs. Extrasynaptic current operates like a filter silencing the whole spinal network, where only pertinent sensory information will be able to pass through and reach supraspinal structures. However, tonic inhibition has a low temporal and spatial resolution whereas fast synaptic inhibition allows better contrast between inputs. The increase we observed in this extrasynaptic current is concomitant with developmental changes in nociceptive circuitry. In newborn rats receptive fields are large and disorganized and mechanical nociceptive thresholds are lower [31]. During postnatal development, mechanical thresholds increase due to a reduction of cell excitability and a change in excitatory and inhibitory synaptic transmission in lamina II neurons [32]. The
increase in tonic GABAergic inhibition that we observed could participate in the postnatal modulation of the balance between excitation and inhibition by setting a higher level of overall inhibition, which in turn decreases the whole network excitability. Therefore nociceptive inputs have to be bigger or better synchronized to override the increased tonic inhibition.

Besides, we propose that high levels of plasma GC and concomitantly increased phasic inhibition lead to a reduction of mechanical pain sensitivity in FIS. These prolonged synaptic inputs are likely to affect temporal integration of excitatory inputs. Nociceptive inputs that are poorly time-locked will hence be compensated by prolonged synaptic inhibition therefore limiting pain processing.

4. Conclusion

Taken together, these results suggest an important role for plasma GC in the spinal regulation of pain processing via 3α5αNS production and their modulatory effects on GABA inhibitory transmission. Since plasma GC levels are under HPA axis control, such a mechanism could provide an additional causal relationship between stress and pain that may be relevant in physiological conditions as well as in pathological states.

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