Research Article

Evaluation and Importance of Amount of Neurosteroids in Nicotine Addiction by Simultaneous Quantification of Neurosteroids in Mice Brain During Nicotinic Sensitization with HPLC

Monappa R. Sutar[1*] and CR. Tenpe[2]

[1]Institute of Pharmaceutical Education and Research, Borgaon Meghe, Wardha - 442 001, Maharashtra, India.

[2]Department of Pharmacology, Institute of pharmaceutical and Education Research, Borgaon Meghe, Wardha - 442 001, Maharashtra, India.

ABSTRACT

Nicotine is one of the major addictive substances consumed by people in the India and world. In order to access the effect of nicotine on content and influence of neurosteroids in brain was studied. The naive mice were used for the nicotinic sensitization with the prior permission of ethical committee. The model for the nicotinic sensitization study used was actophotometer for the measurement of locomotor activity by nicotinic sensitization. Estimation of the brain neurosteroids in mice brain during the developmental phases, withdrawal phase and sensitized phase of nicotinic sensitization were done with the validated high performance liquid chromatography (HPLC). The result obtained evidenced that the neurosteroids play important role in development of nicotine addiction. The result from chromatographic analysis explore that the neurosteroids in brain during nicotinic sensitization increases which may contribute to the development of dependence. Method used above for estimation can be used to investigate the pathological role of neurosteroids and to develop new therapeutics for neurological and psychiatric disorders.

Keywords: Nicotinic sensitization, High Performance Liquid Chromatography, Neurosteroids, Craving.

INTRODUCTION

Nicotine is one of the major alkaloids found in many plants particularly of family *solanaceae* and is a bioactive constituent of tobacco. Tobacco consumption is the principle source of nicotine exposure in human beings. Nicotine is one of the most commonly abused drugs all over the world.[1] Health problems associated with nicotine consumption or smoking is of particular concern. Its chronic consumption leads to a risk for various cancers as well as gastric problems.[2] Nicotine administration produces several pharmacological effects including pleasure, appetite suppression, cognitive enhancement, reduction in anxiety and tension, motor stimulation etc.[3] such effects may contribute to the abuse liability of the tobacco. When repeatedly administered these effects are enhanced and resulted in behavioural sensitization.[1] "**Repeated administration of dependence-producing drugs often results in a progressive enhancement of drug-induced locomotor response This phenomenon, known as behavioural sensitization.**"

The major alkaloid in tobacco, nicotine, activates nicotinic receptors (nAChRs) which increase brain extracellular dopamine producing nicotine reward leading to addiction. nAChRs are located primarily presynaptically and modulate synaptic activity by regulating neurotransmitter release. Subtype-selective nAChR antagonists that block reward-relevant mesocorticolimbic and nigrostriatal dopamine release induced by nicotine may offer advantages over current therapies. An innovative approach has made to provide pharmacotherapies which are antagonists at nAChR & agonist influence at GABAergic receptors subtypes mediating

nicotine evoked dopamine release. Neurosteroids are implicated in various stages of drug dependence, including the acquisition phase, tolerance, and withdrawal.[4,5.] Acute intraperitoneal administration of nicotine or morphine induced dose- and time-dependent increases in the cerebrocortical and plasma concentrations of pregnenolone, progesterone, and allopregnanolone.[6]
Tobacco dependence is the most preventable cause of death and is a chronic, relapsing disorder in which compulsive tobacco use persists despite known negative health consequences.[7,8]

MATERIAL AND METHOD

Subjects

Young healthy male Swiss albino mice weighing 22–24 g were used. They were group housed in a temperature- (22±1 °C) and humidity-controlled (50±5%) environment and had free access to food and water. The animals were treated in accordance with the CPCSEA guidelines for the care and use of laboratory animals and in agreement with the institutional animal ethical committee. The number of animals used and their sufferings were minimized in all experiments designed.
All animal experiments were approved by the Institutional Animal Ethical committee (Registration No. 535/02/a/CPCSEA/Jan2002) of Institute of Pharmaceutical Education and Research, Wardha.

Drugs

The Drugs and other additives used were (-) nicotine hydrogen tartarate, allopregnenolone (allopregnenolone standardd), obtained from Sigma Labs. Saline (0.9% w/v) from medical shop.

Drug solutions and administration

Nicotine free base (Sigma, St. Louis, MO) was dissolved in isotonic saline and administered intraperitonially (i.p.) in a volume of 0.1 ml/mice.

Development of nicotine sensitization

The experiment was carried out in three phases:
1) Seven day development phase (1st to 7th day)
2) Three day withdrawal phase (8th, 9th and 10th day) and
3) Testing phase (11th day).

The mice were injected with nicotine (0.5 mg/kg, i.p.) or saline (0.1 ml, i.p.) for consecutive seven days. During withdrawal phase no treatment was given and on day 11th mice were challenged with same dose of nicotine twice a daily. (Figure 1)
Locomotor activity of animal was evaluated for 20 minutes using actophotometer immediately after administration of first dose of nicotine or saline during all seven days in developmental phase and on day 11 i.e. testing phase. During withdrawal phase locomotor activity was not measured. In SET (II) i.e. control group only saline is administered through the intraperitonial route.

Measurement of locomotor activity

Locomotor activity was measured using actophotometer made up of iron sheet with lid and having digital monitor for activity count with inside 6 laser beams 2.5 cm apart from each other. Animals were kept in actophotometer for about 10 minute's period for adaptation. After the nicotine administration locomotor activity was assessed for 20 minutes.

Measurement of allopregnanolone in brain sample

Level of allopregnenolone in mice brain was measured at the starting of developmental phase on day 0 in saline treated groups (S_{II}) and after day 11 in only nicotine treated groups (S_I).

MATERIALS AND METHOD FOR ANALYTICAL PROCEDURE

Material: Allopregnenolone was purchased from Sigma, St. Louis, MO. Purified water was obtained from Meha Chemicals Water purification system. Methanol (HPLC grade). All reagents were used as received without further purification.

Chromatographic system (High Performance Liquid Chromatography)

In the present study neurosteroid level has been analyzed by HPLC Analysis consisted of a JASCO PU 1580, intelligent pump, a variable wavelength UV-Visible (JASCO UV) detector and a Rheodyne precision loop injector with a 20µl sample loop. Chromatographic separations with UV detection at 245 nm were achieved on a C_8 intersil column (4.6x250mm) (id) with a flow rate of 0.70 ml/min. and ambient temperature. Mobile phases were filtered through a Whatman filter paper No.42 and deaerated under reduced pressure.

Standard solution and calibration

Stock solution was prepared by dissolving 1.0 mg allopregnolone in 10 ml methanol. Standard

solutions were prepared by dilution of stock solution with mobile phase to furnish concentrations in the range 0.1 - 100 µg per ml. Triplicate 10 µl injections of each solution were chromatographed under the conditions described above. Peak areas were plotted against the corresponding concentrations to obtain calibration plot.

VALIDATION OF ANALYTICAL METHOD

Linearity: The linearity of allopregnenolone standards was evaluated by analyzing a set of standards ranging from 0.1 to 100 µg per ml. The calibration curve parameters of allopregnolone showed a linear relationship between peak area and concentration. The mean correlation coefficient, slope and intercept values were 0.9999, 7157, and 282 respectively.

Precision: The percentage RSD of assay of allopregnolone during assay method precision study was within 1 %. The percentage RSD of assay results obtained in intermediate precision study was within 1 % confirming good precision of the method.

Range: The calibration range was established by consideration of the practical range necessary, in accordance with the concentration of allopregnenolone in mice brain, to give accurate, precise and linear results.

Limits of detection and quantification: In accordance with ICH recommendations 23, the approach based on the standard deviation of the response and the slope of the calibration plot was used for determination of limits of detection and quantification. The limit of detection and quantification of allopregnolone was 0.000408 and 0.000938 ng per ml for 10 µl injection volume respectively.

Accuracy: Percentage recovery of allopregnolone in bulk drug samples was ranged from 99.5 to 100.6 %. The excellent recovery obtained suggests the accuracy of the method is good.

Robustness: In all the deliberate varied chromatographic conditions (flow rate, percent organic strength, column temperature), the resolution between allopregnolone and its degradation product was greater than 2, illustrating the robustness of the method.

Solution stability and mobile phase stability

The % RSD of assay of allopregnenolone during solution stability & mobile phase stability experiments was within 1 % RSD. No significant change was observed in the content of allopregnolone during solution stability & mobile phase experiments. The solution stability & mobile phase stability experiments data confirm that allopregnolone sample solutions & mobile phase used during assay were stable for at least 48 hour.

Chromatographic conditions

The choice of chromatographic conditions selected was based on symmetry of peak shape and reduction of chromatographic analysis time. The chromatographic separation was achieved using a mobile phase containing a mixture of aqueous Methanol and Water in the ratio (40:60, *v/v*). In optimized conditions, allopregnenolone and its degradation product were separated with a resolution greater than 2 (Figure 2). The system suitability results were given in Table 1 and the developed HPLC method was found to be specific for allopregnolone and its degradation products.

The chromatogram display only one peak, that of the standard allopregnenolone at 23.0 min (Area under curve=321266). Chromatographic conditions were C_8 intersil column (4.6x250mm), column; mobile phase: methanol–water (40:60); flow-rate: 0.70 ml/min; UV absorbance detection at 245 nm.

Allopregnolone **n =3** determinations (Table 1)

t_R = Retention time in minutes, **Rs** = Resolution

T = Tailing factor, **N** = No. of theoretical plates was close to 100%.

The unaffected assay of allopregnenolone in presence of degradation product confirms the stability indicating power of the method.

The non-interference of allopregnenolone and its degradents confirms the specificity of the developed method in extraction obtained brain samples.

Analysis of neurosteroid allopregnanolone through HPLC in mice brain during the nicotinic development & sensitization phase.[9,10]

In vivo experiment was carried out to test the amount of steroid in brain, during the experimental period on day 0 (before sensitization) and on day 11 (after sensitization). Nicotinic sensitization was done with previous procedure into the mice. The entire brain (1 g) of a male mice (adult age) was collected in a

polypropylene Falcon tube. The sample was then sonicated in an ultrasonic bath for 5 min, left overnight at room temperature and centrifuged at 3000 *g* for 5 min. The organic phase was collected and the rest of the extract residue washed again with 20 ml of MeOH containing 1% CH_3COOH and centrifuged. The two organic phases were pooled and aliquots of 800 ml of the total extract (corresponding to 40 mg of brain tissue) were collected, evaporated to dryness under compressed air in a water bath at 60°C, taken up in 1 ml of MeOH–H_2O (40:60, v/v) and sonicated for 5 min. in a thermo stated ultrasonicator.

A solution of methanol (MeOH) containing 1% acetic acid (CH_3COOH) (10 volumes, w/v) was added together with 1 ug/ml of allopregnanolone. A non-endogenous steroid (Allopregnanolone), was systematically used as the standard for the neurosteroids allopregnanolone.

Previous clean-up step was performed by solid-phase extraction (SPE) on AMPREP silica minicolumns C (500 mg). This 18 C SPE method, previously described by Philippe Liere[a]., Yvette Akwaa., 2000 allows sulfated and unconjugated steroids to be separated.[10]

In the present study neurosteroid level has been analyzed by HPLC Analysis consisted of a JASCO PU 1580, intelligent pump, a variable wavelength UV-Visible (JASCO UV) detector and a Rheodyne precision loop injector with a 20µl sample loop. Chromatographic separations with UV detection at 245 nm were achieved on a C_8 intersil column (4.6x250mm) (id) with a flow rate of 0.70 mL/min. and ambient temperature. Mobile phases were filtered through a Whatman filter paper No.42 and deaerated under reduced pressure.

Samples were deposited on columns which had been previously activated successively with 5 ml of MeOH, 5 ml of H_2O and 5 ml of 2 MeOH–H_2O (40:60, v/v). The unconjugated steroid fraction was eluted with 5 ml of MeOH–H_2O (85:15, v/v), filtered through a 0.45-mm Millipore PFTE membrane and dried.

Formula for determination of concentration of unknown sample

$$\textbf{Concentration of Unknown Sample} = \frac{\textbf{Conc. of Standard X Peak area of Sample}}{\textbf{Peak area of Standard}}$$

Statistical Analysis

Statistical analysis was carried out by One Way analysis of variance (ANOVA) followed by post hoc comparisons using Dunnett Test. $P \le 0.05$ was considered to be statistically significant.

RESULTS

Nicotinic Sensitization

Locomotor activity counts for 20 minutes after injections of nicotine produced significant increase in locomotor activity, compared with those saline treated mice. (***p<0.05), reflecting that sensitization to nicotine was produced and persisted on day 11. (Figure 3)

Each value represents the mean ± SEM (n=6)

Stastical analysis performed by One Way ANOVA with post hoc Dunnett Test ($p<0.05)

***P<0.05 Saline treated groups compared with nicotinic groups.

Analysis of neurosteroid allopregnanolone through HPLC in mice brain during the nicotinic development & sensitization phase.[5,6]

(figure number 2)

The chromatogram display only one peak, that of the standard allopregnenolone at 23.0 min (Area under curve=321266). Chromatographic conditions were C_8 intersil column (4.6x250mm), column; mobile phase: methanol–water (40:60); flow-rate: 0.70 ml/min; UV absorbance detection at 245 nm.

Results of the chromatogram are depicted in table and figure. (Refer table 2 and Figure 4)

DISCISSION

The locomotor response to a 0.5 mg/kg dose of Nicotine for Sensitization in mice was the same as repeatedly observed in previous studies.[1] The most prevalent hypothesis of nicotine addiction includes a scenario where nicotine first stimulates DA release in neurons from the ventral tegmental area (VTA), promoting long-term potentiation of inputs onto dopaminergic neurons.[6,9] The DA-producing neurons originated in the VTA extend to DA-sensitive cells located in the nucleus accumbens. This dopaminergic pathway is neuroanatomically part of the mesocorticolimbic system which is functionally considered as the "brain reward

circuitry". Drugs of abuse block DA reuptake (e.g., cocaine) and/or increase DA release (e.g., nicotine) in VTA terminals, enhancing DA signaling in the nucleus accumbens and thereby promoting addiction behavior.[15]

In this experiment in both sets period of locomotar response of 10 minutes has done for adaptation of experimental condition. Locomotar response has been produced by treatment of nicotine (0.5 mg/kg ip). The schedule followed was development phase of seven days (1st to 7th), withdrawal phase of three days (8th,9th,10th day), & testing phase (on day 11). As regards with experiments it include, the animals got exposure of challenge dose of nicotine (on day 11) with the same dose of nicotine (0.5 mg/kg ip) two times a day. Nicotine has been shown to cause great changes in psychomotor reactivity and mesolimbic dopamine activity.[1] However, in this experiment there was case as significant difference was found between the locomotor response to saline (control) and nicotine-treated animals.

To further we estimated neurosteroid level in mice brain.[2,5,12,13,] In this investigation before starting study, after nicotine treatment neurosteroid estimation was done. Results indicated that neurosteroid level in brain has been increased after nicotinic sensitization. The result from chromatographic analysis (HPLC) explore that the neurosteroids in brain during nicotinic sensitization increases which may contribute to the development of dependence by GABAergic influence. Comparision was done in saline treated and Nicotne treated groups of mice. (One way ANOVA **P<0.005). The results are in consistent with previous investigation that corticosteroids modulate responsiveness of mice & rat to nicotine. The probable mechanism involved by acting on afferent or efferent nAch receptor, or decreased responsiveness to nicotine action reduces nicotine access to nAch receptor, or desensitization of nAch receptor, or recently suggested allosteric inhibition of nAch receptor.[9]

Table 1: System suitability report

Compound (*n*=3).	t_R	RS	N	T
Allopregnolone	23	11.2	8970	1.09

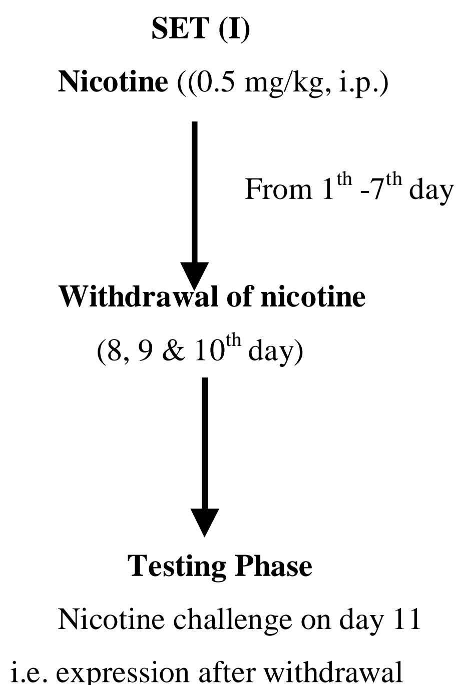


Fig. 1: Diagrammatic representation of the method for nicotinic sensitization

Table 2: Effect of nicotinic sensitization and muscimol treatment on levels of allopregnanolone in mice brain

S. NO.	AUC of normal mice (concentration/gm)	AUC of nicotinic sensitized mice (concentration/gm)
1	17120 (0.532 ng/gm)	22137 (0.689 ng/gm)**
2	14427 (0.449 ng/gm)	24170 (0.752 ng/gm)**
3	13117 (0.408 ng/gm)	29271 (0.911 ng/gm)**
4	15002 (0.466 ng/gm)	30140 (0.938 ng/gm)**
5	18170 (0.565 ng/gm)	27907 (0.868 ng/gm)**
6	21237 (0.661 ng/gm)	25557 (0.795 ng/gm)**

(One way ANOVA, Dunnett test) **P<0.05

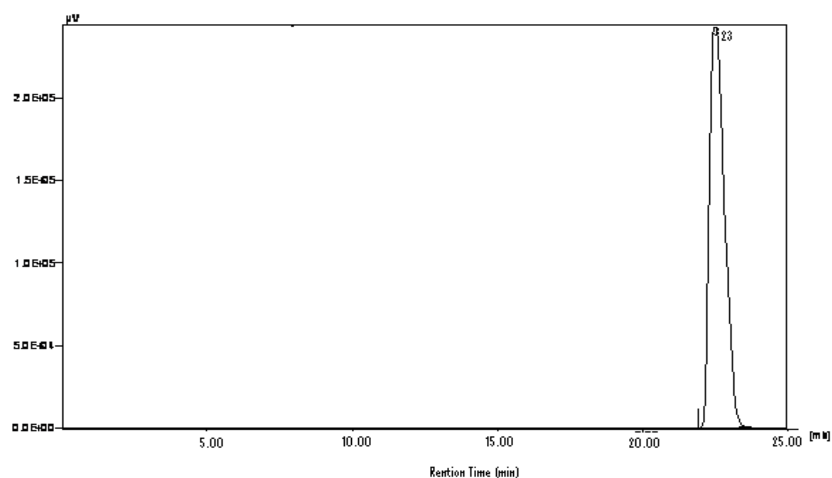
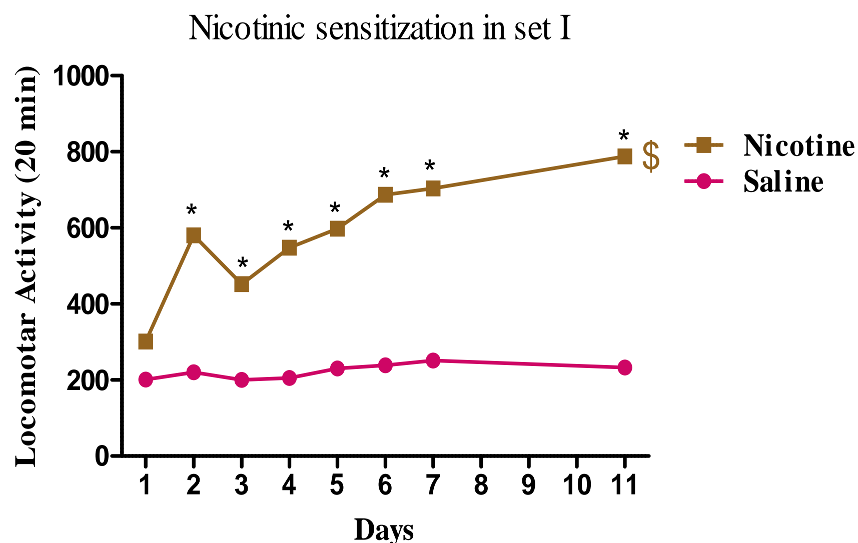


Fig. 2: Chromatogram obtained from the injection of 1ug/ml of allopregnanolone



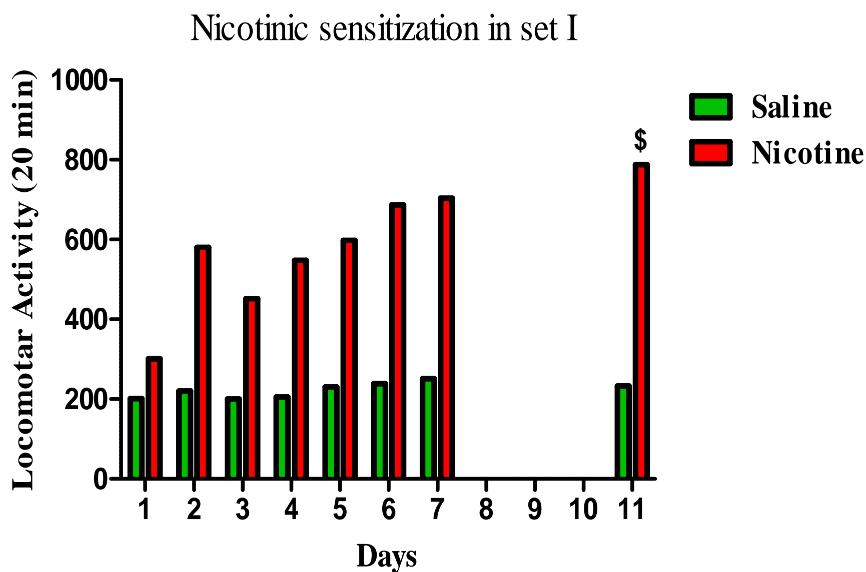


Fig. 3: Nicotinic sensitization

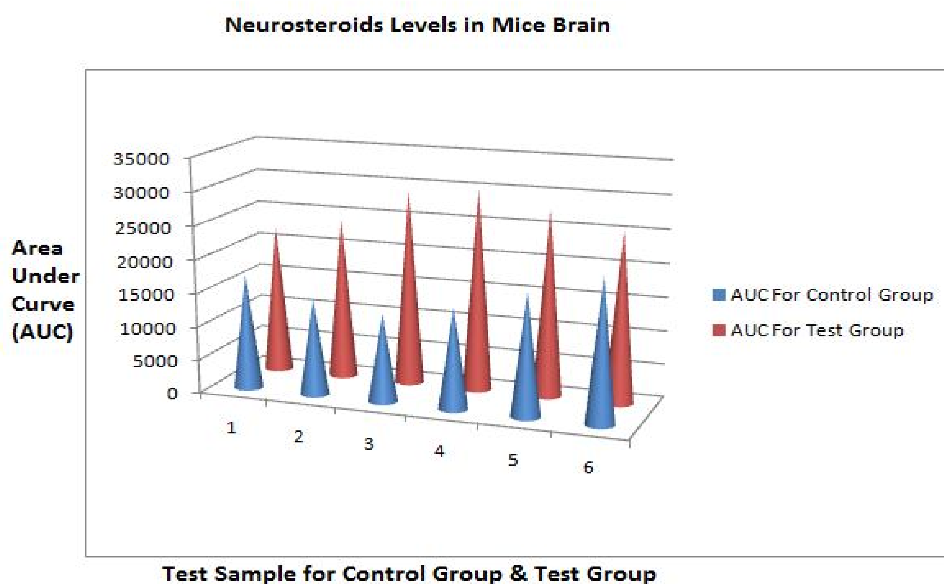


Fig. 4: Neurosteroid level during normal, nicotinic sensitization

CONCLUSION

From the above study it is concluded that nerosteriods play important role during nicotine addiction. Dependance phenomenon is characterized by the craving of nicotine. So in coordination with the previous study it is found that neurosteroids increases during nicotinic sensitization. Neorosteroids are the allosteric modulators of the of GABAa receptors by the mean these are executing the inhabition of

nicotine dependence. Due to the abuse of nicotine there may less production of neurosteroids which eventually leads to the craving and dependence.
So, in coordination with the previous study exogenous administration of the neurosteroids may decrease the nicotine dependence by allosteric modulation of the Gabargic receptors in brain.
The chromatographic method applied above may serve as economic and time saving method for the investigation of pathological role of neurosteroids and to develop new therapeutics for neurological and psychiatric disorders.

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