Research report

Striatal preprotachykinin mRNA levels are regulated by stimulatory agents and dopamine D1 receptor manipulation in rodent organotypic slice cultures

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Abstract

We have utilized an organotypic slice culture system to determine factors which directly influence the expression of striatal preprotachykinin (PPT) mRNA. Striatal slices were generated from 3-day-old male rat pups and cultured on Millicell-CM inserts in serum-containing media. Under these conditions, striatal PPT mRNA levels fell significantly (\(-55.7\pm6.2\%\)) in slices cultured for 2 days in vitro (2DIV) as compared to slices placed in culture for 3 h (0DIV). However, striatal PPT mRNA expression did not decline further in 4DIV cultured slices (\(-59.6\pm7.1\%\)). When 2DIV slices were exposed to combined high potassium (\(K^+\), 10 mM) and forskolin (10 \(\mu M\)) stimulation for 3 h, PPT mRNA levels were increased within areas of the brain normally associated with tachykinin production. Application of the dopamine (DA) D1 receptor agonist SKF-38393 (10 \(\mu M\)) at 2DIV for 3 h also increased (\(+162.9\pm28.9\%)\) PPT mRNA expression, but increases were localized within the striatum. SKF-38393-stimulated increases were completely blocked by the D1 antagonist SCH-23390 (10 \(\mu M\)), which alone had no effect on mRNA levels. However, a 3-h incubation with SKF-38393 on 0DIV slice cultures did not affect PPT mRNA expression whereas SCH-23390 decreased PPT message levels (\(-24.5\pm5.4\%)\). These findings indicate that tachykinin gene expression is inducible within slice culture preparations and that the maintenance of normal striatal PPT mRNA levels depends on DA D1 receptor tone.

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1. Introduction

Our current understanding of striatal tachykinin gene regulation by trans-synaptic factors is based largely upon whole animal studies involving systemic drug administration. Although such approaches have contributed valuable information to the field of basal ganglia research, data interpretation problems inherent to these models have necessitated the need for alternative experimental systems where variables can be more easily controlled and manipulated. The organotypic slice culture model developed by Stoppani et al. [29] provides an additional approach in which to study how different receptor systems interact to regulate striatal transmission. This method permits drug application directly to striatal cells maintained in an in vitro environment which retains much of the intrinsic organization of the intact striatum. For example, cholinergic interneurons are present in organotypic slice cultures [1,10] indicating that the intrastriatal cholinergic network may still be functional. This is important because cholinergic interneurons may influence how dopamine (DA) receptor systems interact to regulate tachykinin biosynthesis [32]. Also, at least a portion of the glutamatergic innerva-
tion from the cortex remains functionally connected in slice cultures [30] and glutamate receptor stimulation influences the transcription of striatal preprotachykinin (PPT) mRNA [27,28]. Furthermore, D1 receptors have been reported to regulate the expression of transcription factors in organotypic slice cultures [17] which are important in the activation of striatal tachykinin gene expression.

How DA receptor populations interact to regulate striatal tachykinin biosynthesis remains unclear. In the intact striatum, blockade of either D1 or D2 receptors reduces striatal PPT mRNA expression levels [2,3,13] indicating that DA transmission is involved in stimulation of tachykinin synthesis. Furthermore, in DA-depleted animals, PPT message levels are reduced [24,25,33] and stimulation with SKF-38393, a D1 receptor partial agonist, reverses this trend [5,11]. However, stimulation of D1 receptors within the intact striatum with the full agonist, SKF-82958, has been reported to increase PPT message levels [32] while partial agonists do not [13]. Also, stimulation of D2 receptors may positively regulate PPT mRNA expression, but this mechanism seems to be dependent on D1 receptor tone [13].

In the present study, we have examined the relationship between D1 receptor activation and the maintenance of tachykinin gene expression within striatal organotypic slice cultures. The use of this system is advantageous in that it allows brain tissue to be exposed to a known concentration of drug without the pitfall of diffusion which occurs following direct brain injections. Also, it limits the influence of indirect mechanisms where D1 receptor stimulation could regulate PPT message levels such as activation of distant anatomic circuits that innervate the striatum. The results of this study are consistent with the idea that D1 receptor stimulation is necessary for basal tachykinin gene expression within the striatum. Furthermore, it indicates that cultured striatum responds similar to DA lesion models in that PPT message levels decline and D1 receptor activation reverses this trend.

2. Materials and methods

2.1. Organotypic slice cultures

Postnatal day 3 male Sprague–Dawley rat pups (Charles River Laboratories) served as the origin for organotypic slice cultures in all experiments with the exception of one in which postnatal day 5 animals were used as age-matched controls. Three to four coronal sections (300 μm) per hemisphere were obtained using a Campden Instruments vibratome starting at −2.0 mm from Bregma [23] and collected in ice cold oxygenated Ringer’s solution containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1.0 NaH2PO4, 26.2 NaHCO3, and 2.0 g/l D-glucose. Slices were rinsed 3× in cold Minimal Essential Media (MEM; Gibco-BRL), and transferred to 0.4-μm Millicell-CM (Millipore) tissue culture inserts. The inserts were incubated at 37°C, 5% CO2 in media which contained 50% MEM, 25% Hank’s Balanced Salt Solution (Gibco-BRL), 25% heat-inactivated horse serum, 2 mM NaHCO3, 2 mM l-glutamine, and 6.5 mg/ml D-glucose [29] supplemented with 10 ng/ml brain-derived neurotrophic factor (BDNF; Sigma). At 1 day in vitro (DIV), the media was replaced with fresh media containing 100 U/ml penicillin, 100 mg/ml streptomycin but devoid of BDNF. Slices were maintained in culture for 2 or 4 days in vitro (DIVs). Some slices were terminated at 3 h after initial culturing (0DIV).

All animal use procedures were in strict accordance with the NIH Guide to the Care and Use of Laboratory Animals and approved by Wayne State University Animal Investigation Committee.

2.2. Drug treatments

At 2DIV, slices were exposed to vehicle or drug treatment for 3 h. For high K+/forskolin (K/For) stimulation, slices received a final concentration of 10 mM K+ and 10 μM forskolin (Sigma) [31] dissolved in a vehicle of MEM–dimethylsulfoxide. SKF-38393 (Sigma) and SCH-23390 (RBI) were each administered at 10 μM [17] in a vehicle of 0.9% saline. Cultures were harvested by immersion in 4% paraformaldehyde. Where possible, control and treatment could regulate PPT message levels such as activation of drug without the pitfall of diffusion which occurs following direct brain injections. Also, it limits the influence of indirect mechanisms where D1 receptor stimulation reverses this trend. 

2.3. ‘Free-floating’ in situ hybridization

PPT cRNA probes were generated from the plasmid pGEM2-31-1 containing the full-length 1100 nt PPT cDNA [15]. The resulting 35S-UTP labeled antisense PPT message levels are reduced [24,25,33] and stimulation with SKF-38393, a D1 receptor partial agonist, reverses this trend [5,11]. However, stimulation of D1 receptors within the intact striatum with the full agonist, SKF-82958, has been reported to increase PPT message levels [32] while partial agonists do not [13]. Also, stimulation of D2 receptors may positively regulate PPT mRNA expression, but this mechanism seems to be dependent on D1 receptor tone [13].

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changes between sections were calculated by measuring the average optical density of the entire striatal region, the ventral thalamus (ventrolateral, ventromedial, ventral posterolateral, and ventral posteromedial), the ventromedial hypothalamus and a fixed circular area within the frontal cortex. Background noise was determined by measuring optical density values in the corpus callosum and subtracted from all data. Significance ($P<0.05$) was determined by Student’s t-test or one-way analysis of variance (ANOVA) with Tukey-HSD post hoc using SPSS 8.0 statistical software.

3. Results

Slice cultures were analyzed for PPT mRNA content from 0DIV to 4DIV. At 0DIV PPT mRNA expression was observed in the striatum, thalamus, hypothalamus, amygdala, and areas of the cortex. Expression was also seen to a lesser extent in the hippocampus. Levels of PPT mRNA were reduced to 44.3±6.2% by 2DIV in the striatum, but these levels were maintained at 40.4±7.1% at 4DIV (Fig. 1).

The responsiveness of the slice culture paradigm was tested with K/For at 2DIV. Acute 3 h stimulation resulted in increased (+63.9±15.4%) striatal PPT message levels (Fig. 2). Levels of gene expression were also increased in the thalamus, hypothalamus, and areas of the cortex indicating PPT mRNA expressing cell types in several brain regions remain responsive to K/For stimulation at 2DIV.

Acute D1 receptor stimulation at 2DIV with SKF-38393 caused a significant increase (+162.9±28.9%) in striatal PPT mRNA expression (Fig. 3). This D1 agonist effect was completely blocked by SCH-23390, a D1 antagonist which alone had no effect on PPT mRNA expression (Fig. 3B). SKF-38393 did not significantly alter PPT mRNA levels in any other region examined indicating its specificity for PPT gene regulation within the striatum (Fig. 3C). However, acute stimulation with SKF-38393 did not alter PPT mRNA expression in 0DIV brain slices, whereas blockade of D1 receptors by SCH-23390 produced a small but significant decrease in PPT mRNA expression in the striatum with ($-25.9±4.4%$) or without ($-24.5±5.4%$) the addition of SKF-38393 (Fig. 4). We also analyzed PND5 slices at 0DIV to compare PND3 2DIV slice cultures with age-matched controls. Application of SKF-38393 and SCH-23390 to PND5 0DIV cultures produced results identical to PND3 0DIV cultures (data not shown) suggesting that the differences in D1 receptor stimulation at 0DIV and 2DIV in PND3 animals were not a result of a developmental change in PPT mRNA expression.

To determine the extent to which D1 receptor stimulation was able to rescue PPT mRNA expression, 0DIV cultures were directly compared to 2DIV cultures with or without the addition of SKF-38393. Stimulation with SKF-38393 in 2DIV cultures increased PPT mRNA in the striatum to nearly (89.1±10.1%) 0DIV control levels (Fig. 5). Taken together, the results of these experiments suggest that an existing level of D1 receptor tone is necessary to maintain basal expression of striatal tachykinin production.

4. Discussion

The purpose of this study was to examine tachykinin gene regulation by D1 receptor stimulation within striatal tissue maintained in vitro. Our results agree with previous findings that PPT mRNA expression is reduced under slice culture conditions [14]. However, application of non-specific stimulating agents (K/For) increased PPT mRNA levels at 2DIV indicating that tachykinin neurons remain viable and responsive to broad stimulating compounds in culture. It is interesting that while D1 receptor stimulation was ineffective in raising PPT mRNA levels during the initial hours of culturing, D1 receptor antagonism lowered striatal PPT mRNA expression. However, D1 receptor stimulation significantly increased PPT mRNA levels at 2DIV but the effects were localized within the striatum. Together, these data indicate that D1 receptor tone is necessary for basal expression of PPT mRNA within the striatum.

Why PPT mRNA levels are reduced in culture over time remains to be determined, although several factors may explain this phenomenon. Among these is the loss of monoamine innervation since projections to the striatum from the substantia nigra and dorsal raphe are axotomized during culture preparation. However, the removal of 5-HT afferents in intact or DA depleted rodents does not appear to significantly alter PPT mRNA expression [4]. Therefore, it is more likely that the loss of DA in our culture system may produce the observed reduction in PPT mRNA content rather than the loss of 5-HT. It is well established that PPT gene expression and substance P content are...
Fig. 2. Effects of non-specific stimulating agents on PPT mRNA expression. (A) Autoradiograph representing 2DIV slice cultures with or without K/For stimulation. (B) Densitometric analysis of PPT mRNA levels in the striatum (str) at 2DIV demonstrated that application of high K⁺ (10 mM) and forskolin (10 μM) resulted in increased gene expression as compared to untreated control cultures (*: P<0.05 compared to untreated control, Student t-test). Each group represents at least five cultures in duplicate. PPT message levels were also significantly increased in the thalamus (+52.98±26.27%; thal), hypothalamus (+49.87±14.02%; hyp), and cortex (+84.52±21.44%; ctx).

depressed in the striatonigral pathway of DA depleted rodents [16,18,24,26,33]. Also, DA antagonists in intact rodents decrease PPT message levels and substance P content in striatonigral neurons [2,3,12]. Although it is unknown how long DA terminals remain viable within the organotypic slice culture model, preliminary high-performance liquid chromatography–electrochemical detection (HPLC–ED) analysis shows that both DA and 5-HT levels are severely depressed to <10% at 2DIV [9]. Moreover, while the total number of striatal projection neurons surviving in organotypic slice culture is expected to be roughly 60–70% [17], we have yet to establish the relative loss of PPT mRNA containing neurons in culture. Therefore, cell death is also expected to contribute to a portion...
Fig. 3. Striatal PPT mRNA levels respond to D1 receptor stimulation at 2DIV. (A) Autoradiograph representing 2DIV slice cultures 3 h following vehicle, 10 μM SKF-38393, 10 μM SCH-23390, or combined SKF/SCH application. (B) Densitometric analysis of PPT mRNA levels in the striatum in response to D1 agonism/antagonism. PPT mRNA expression was increased only within the striatum (arrow) of cultures treated with SKF-38393 alone. This response was completely blocked by SCH-23390 that alone had no effect. (C) Regional densitometric analysis of PPT mRNA in response to D1 agonism at 2DIV. Increases in PPT mRNA levels are localized to areas associated with high concentrations of D1 receptors (*: P<0.05 compared to untreated control cultures, #: P<0.05 compared to SKF-38393 treatment, one-way ANOVA with Tukey-HSD post hoc test). All groups represent mean±S.E.M. of at least five cultures in duplicate.
of the reduced PPT mRNA content within striatal slice cultures.

Even though PPT mRNA levels decline in the organootypic slice culture environment, tachykinin neurons remain responsive to the application of stimulatory agents. Previous studies have shown that K⁺ depolarization in combination with direct activation of adenylyl cyclase by forskolin produced moderate increases in activation of the PPT promoter [21,31]. In our experiments, stimulation with K⁺ for increased PPT mRNA levels throughout the slice culture indicating that transcription of the tachykinin gene remained functional in a variety of cell types includ-

ing tachykinin neurons of the striatum. However, it is interesting to note that while application of non-specific stimulating agents increased PPT mRNA in all areas of the brain normally expressing PPT message, D1 receptor-induced stimulation of PPT expression was targeted primarily to the striatum. This would be predicted since D1 receptors are located within the striatum with little or no expression in other tachykinin producing regions examined [20,22].

The stimulation of striatal PPT mRNA levels in response to D1 receptor activation at 2DIV indicates that DA receptors remain functionally coupled to tachykinin production in culture. However, the lack of effect by SKF-38393 in both PND3 and PND5 0DIV cultures suggests that alterations occur in the culture environment over time that are necessary to observe the effects of D1 stimulation. An explanation for these results could be that D1 receptor tone is necessary for basal expression of PPT mRNA. This would be consistent with observations from experiments performed in vivo which show that SKF-38393 does not affect PPT mRNA levels prior to depletion of DA [5,13]. As such, it is possible that 0DIV cultures, unlike 2DIV cultures, retain enough endogenous DA to maximally stimulate D1 receptors. In support of this theory, DA content within the striatum of 0DIV cultures is ~85% of uncultured tissue [9]. Moreover, PPT message levels are reduced following D1 antagonism at 0DIV with no effect at 2DIV. Also, direct comparison of message levels in 0DIV and 2DIV cultures indicates that D1-induced striatal PPT mRNA expression is not an increase in gene activation, but instead a return to baseline. Together these data support the idea that tonic D1 receptor activation is necessary for basal PPT mRNA expression.

However, as previously noted, some cell loss is expected in culture. Similar slice culture experiments have estimated that 60–70% of DARPP-32 positive cells survive in culture for 3 days [17]. Unfortunately, it is unknown what portion of these cells are tachykinin producing neurons and which contain enkephalin. As such, it is entirely plausible that a small population of tachykinin producing neurons remains in culture and becomes super-sensitized to D1 stimulation. This would be consistent with whole animal studies in which activation of D1 receptors following DA depletion resulted in increased cAMP and c-fos production without changes in D1 receptor concentration or binding [6–8,19]. Therefore, the response we measured may in fact result from individual cells expressing increased levels of PPT mRNA rather than a regional return to baseline. Whether the effects of D1 stimulation on PPT mRNA expression in this study were due to receptor plasticity resulting in strengthened gene regulation or simply caused by the replacement of a tonically active stimulatory influence on striatal neurons has yet to be determined. Further studies will be necessary to distinguish between these possibilities. In either case, though, it seems likely that D1 receptor...
stimulation is necessary to maintain striatal PPT mRNA expression in organotypic slice cultures.

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