

Serotonin receptor activation enhances neurite outgrowth of thalamic neurones in rodents

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Received 17 February 1999; received in revised form 7 May 1999; accepted 10 May 1999

Abstract

Serotonin (5-HT) has been shown to influence the development of the rodent barrel field by affecting the patterning of thalamic axons in the somatic sensory cortex. To determine whether this is a direct effect on thalamocortical neurones, we analyzed primary thalamic cultures taken from E15 mouse embryos. We show that 5-HT enhances neurite outgrowth of thalamic neurones. The sodium channel blocker, TTX, blocks these effects, whereas the selective 5-HT_{1B} agonist CGS-12066A maleate reproduced 5-HT's effect. Using PCR and immunocytochemistry, we found that 5-HT_{1B} receptors are already expressed by thalamic neurones at E15, and that this expression is maintained *in vitro*. These results suggest that 5-HT_{1B} receptor activation directly affects the growth of thalamocortical axons. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Serotonin; Neurite outgrowth; Thalamic neurones; Rodent barrel field

In addition to its role in neurotransmission, serotonin (5-HT) regulates many aspects of development including neuronal growth and survival of neurones [9,11]. Of particular relevance to the present analysis, 5-HT has been shown to affect the fine-tuning of thalamocortical connections. For instance, the formation of barrels in rodents is disturbed by modifying brain levels of 5-HT during development [2,4]. In the latter case, changes in the size of individual barrels has been correlated with changes in thalamocortical growth in cortical layer 4, suggesting that 5-HT promotes the growth of thalamic axons during development. Although developing thalamic neurones transiently express 5-HT_{1B} receptors in rats and mice [1,4] and the plasma-membrane transporters of 5-HT, SERT, [10], it is not clear that 5-HT acts directly on thalamic neurones. 5-HT could also act indirectly by causing changes in the growth-promoting properties of intermediate targets, such as cortical neurones or glial cells that also contain serotonin receptors of the 5-HT₁ and 5-HT₂ type [15,20]. Furthermore, since changes in neural activity affect the growth of thalamic axons [14], it is not clear whether 5-HT's effects are mediated by a change in neural activity, or by a direct effect on growth. To answer these questions we used dissociated primary cultures of embryonic thalamic neurones, a

preparation that contains almost exclusively the neuronal population of interest.

The culture protocol used here has been previously described in detail [13]. Following this protocol, cells were plated at 500 cells/mm², in 96 well cluster plates (Costar) in a defined serum free medium. 5-HT (36 pM, 3.6 nM, or 0.36 μM), TTX (1 μM); CGS-12066A (1.7 nM), or phenylbiguanide (4 nM) were added to the culture medium from the outset. After 24 h, the cultures were fixed in 4% formaldehyde. For PCR, total RNA was extracted from unfixed tissue cultures after 24 h. Cell viability was determined by assessing nuclear morphology with bisbenzimidazole (10 μg/ml Hoescht 33342, Sigma). The nuclei of viable neurones are large, diffusely labelled; nuclei of dead/dying cells are small and brightly labelled [13]. Consistent with our previous observation [13], 27% of plated thalamic neurones were viable at 24 h, and only 8% after 3 days in culture. All surviving cells were labelled with MAP2 antibodies, and no GFAP positive staining was observed. The addition of 5-HT to the culture medium did not change the survival rate. Immunocytochemical localization with 5-HT_{1B} antibodies (a kind gift of Michel Hamon) showed that most of the cultured thalamic neurones (plated at 500 or 2000/mm²) expressed this receptor subtype (Fig. 1B). The presence of this specific receptor subtype was further confirmed by PCR analysis showing the presence

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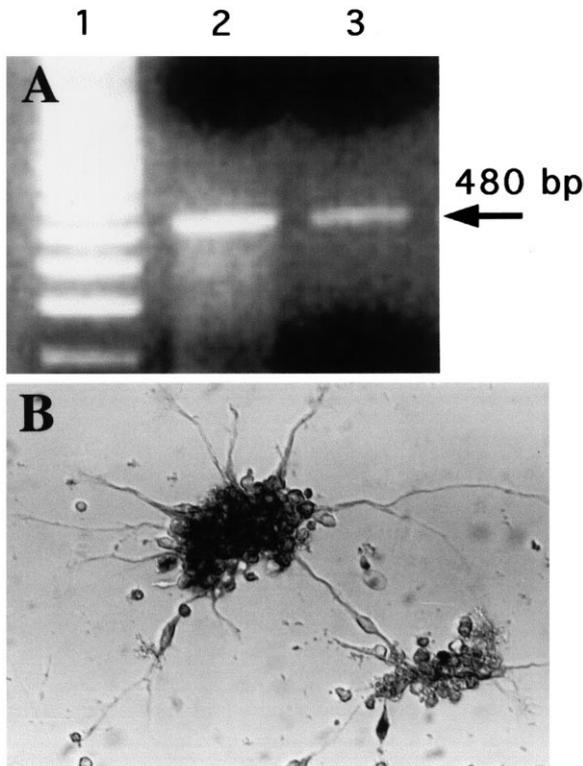


Fig. 1. (A) Gel of RT-PCR reaction products using primers specific for 5HT-1B with RNA isolated from cell cultures of the raphe and thalamus taken at E15 after 1 day in vitro (DIV); the reaction products were separated on a 1% agarose-TAE gel. Lane 1: 100 base pair-ladder, molecular weight markers. Lane 2: RT-PCR product from RNA from an E15 plus 1 DIV culture of raphe neurons from an OF1 mouse. Lane 3: RT-PCR product from RNA from an E15 plus 1 DIV culture of thalamic cells from a C3H/HeJ mouse. (B) 5-HT1B immunocytochemistry on E15 thalamic cultures (plating density 2000/mm²) after 1 DIV, neurites and neurones emerging from the neuronal aggregate display specific immunostaining ($\times 291$).

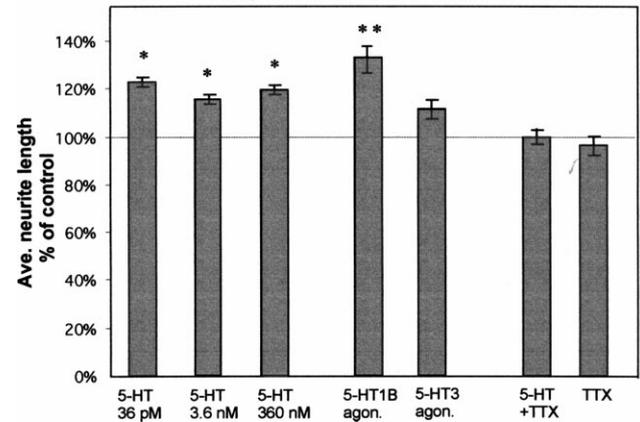


Fig. 3. Graphs showing the average neurite length of E15 thalamic neurons after 22–24 h in culture with different concentrations of 5HT, 5HT-1B or 5HT-3 agonists. Measures of neurite length were done under phase contrast, with a $\times 32$ objective. Ten 0.13×0.13 mm windows were randomly selected and captured with a video camera. All neurons in which the tip of the longest neurite could be localized were included. Most neurites were tipped with growth cones. The length of the process was measured from the cell soma up to the leading edge of the growth cone's lamellipodia, not including the filipodia. The values are expressed as percentages of the neurite length (26 μ m) in control conditions (\pm SEM). Significant increases were determined using Student's *t*-test, and are noted ($*P < 0.01$).

of 5-HT1B mRNA (Fig. 1A). Using this same approach we determined that cultured E15 thalamic cells also express the plasma membrane transporter of serotonin (SERT) (data not shown) as in vivo [10].

After 22–24 h in vitro, all the viable thalamic neurones extended one to three neurites in the control and 5-HT supplemented medium. One of these neurites was consistently longer than the rest. The mean length of this longest neurite was $26 \pm 0.3 \mu$ m in the control condition (Fig. 2a). The addition of 5-HT to the culture medium significantly

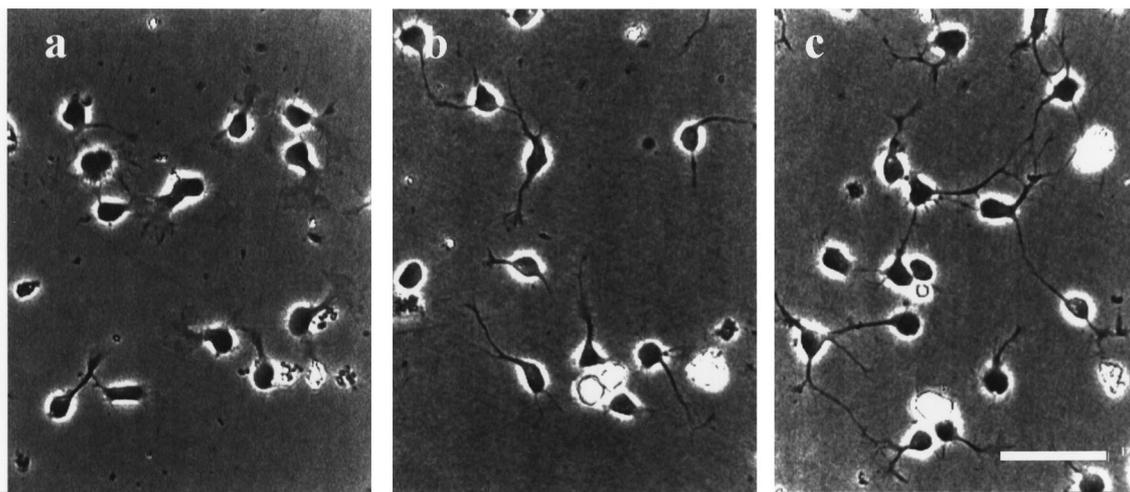


Fig. 2. Photomicrographs of E15 thalamic neurons cultured for 22–24 h, phase contrast micrographs of cultures grown (a) alone, (b) with 3.6 nM 5HT, or (c) with 2.5 nM 5HT-1B agonist. It is apparent that 5HT and the 5HT-1B agonist enhance neurite extension whereas neurite branching and cell diameter were not noticeably affected. Scale bar, 50 μ m.

increased neurite outgrowth (Fig. 2b) under all concentrations tested (Fig. 3) in the order of 17–23%. Increasing the concentration of 5-HT above 0.36 μM decreased the average neurite length and ultimately reduced cell viability.

To determine which 5-HT receptor subtype is involved in the neurite growth-promoting effect, we tested different agonists of 5-HT receptors (Fig. 3). CGS-12066A Maleate (1.7 nM), a specific 5-HT_{1B} agonist, had pronounced growth-promoting effects (Fig. 2c). The growth induced by the 5-HT₃ receptor agonist 1-phenylbiguanide, though above control levels, was significantly less than that induced by the 5HT-1B agonist. To test whether the growth enhancing effects of 5-HT are activity-dependent, the sodium channel blocker tetrodotoxin (TTX) was added to the culture medium. TTX in itself did not affect the average length of thalamic neurites but inhibited the growth-promoting effects of 5-HT (Fig. 3).

The present results complement and extend previous findings *in vivo* that showed an effect of 5-HT on the development of thalamocortical axonal projections in the rodent barrelfield [2,4]. Within each cortical barrel, thalamic axons form a distinctive cluster that corresponds to sensory afferents related to one vibrissa. The size of these thalamocortical clusters is reduced by 20% when serotonergic afferents are damaged at birth [2], whereas increased levels of 5-HT in the brain during early postnatal life led to an expansion of cortical barrels and eventually to their complete fusion in MAOA knockout mice [4]. In these experimental situations, besides the alterations of the thalamocortical projection, there are changes in the organization of target cortical neurones and in the distribution of tenascin [4], a molecule that is produced by glial cells, and that could act on axon growth. It is therefore difficult to determine whether 5-HT acts primarily on the thalamic neurones themselves or indirectly via cortical neurones or glia. Our primary thalamic cultures indicate that 5HT *in vivo* could act directly on thalamic neurite extension.

Previous *in vitro* studies have shown that 5-HT modulates cell maturation or neurite outgrowth. However the effects varied substantially among the cell types and systems that were analyzed. For instance, in the sphinx moth exposure to 5-HT enhances neurite growth of subpopulations of antennal lobe neurones [16], whereas in the snail (*Helisoma Trivolvis*) 5HT arrested neurite outgrowth [5,6]. Similarly in the rat brain, 5-HT's effects varied according to the cell type that was analyzed. In the cerebral cortex, 5-HT_{1A} receptor activation caused a reduction of neurite extension and branching [19] while 5-HT₂ receptor activation increased the dendritic differentiation of calretinin positive neurones [8]. 5-HT also promoted the differentiation of cholinergic [18] and monoaminergic neurones [12]. The variability of these recorded effects makes the point that 5-HT's effects can differ considerably according to the receptor subtypes that are activated and the coupled transduction mechanisms. In the E15 embryonic thalamic neurones that we studied, our results suggest that 5-HT's

neurite outgrowth promoting effect is mediated by 5HT-1B receptors for the following reasons. 5HT-1B receptors are transiently expressed in thalamocortical projections during postnatal life [1,4]; they are generally located pre-synaptically [3] and exert an inhibitory effect electrophysiologically, possibly by decreasing the release of glutamate [17]; their expression begins as early as E15 in mice, and is maintained *in vitro*; and, selective activation of the 1B receptor subtype is most effective in enhancing thalamic growth. The fact that 5-HT's effects are observable at the lowest concentrations studied (in the picomolar and nanomolar range) is also consistent with the high affinity of the 5-HT_{1B} receptor subtype [7]. As thalamic growth is regulated by activity in culture [14], one strong possibility is that 5-HT acts on this system by altering neural activity. Consistent with this suggestion, co-adding 1 μM TTX with 5-HT eliminates the growth promoting effects of 5-HT, demonstrating the requirement for activity for the growth promoting effects of 5-HT.

We are grateful to Gail Lotto and Chantal Alvarez for their assistance. This project was supported by INSERM and the European Commission (BMH4CT97–2412).

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