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Does the endogenous opiate system play a role in the Restless Legs Syndrome?: A pilot post-mortem study

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ABSTRACT

Opioids are an effective treatment for the signs and symptoms of Restless Legs Syndrome (RLS) and the signs and symptoms of RLS return when the opiate receptor blocker naloxone is given to opioid treated RLS patients in a blinded fashion. These data suggest that the opioid effect is specific to the opiate receptor in RLS and implicate the endogenous opioid system with its enkephalins and endorphins in the pathogenesis of RLS. We therefore measured Beta endorphin, Met-enkephalin and Leu-enkephalin levels in thalamus and substantia nigra of RLS patients (5 F — avg age 80.2 years) compared to controls (5 F, 1 M — avg age 76.3 years). One half of each brain was fixed in paraformaldehyde (PFA) in phosphate buffered saline (PBS) for pathologic evaluation and paraffin sections were stained with antibodies. Cell numbers were counted in a blinded fashion. In the thalamus, there were reductions of Beta-endorphin and Met-enkephalin positive cells by 37.5% ($p=.006$, effect size 2.16) and 26.4% ($p=.028$, effect size 1.58), respectively, in RLS patients compared to controls. There was no difference in Leu-enkephalin in the thalamus or changes in Beta endorphin, Met-enkephalin, Leu-enkephalin or Tyrosine Hydroxylase, the rate limiting step for dopamine synthesis, in the substantia nigra. Although one of the main hypotheses for pathogenesis has been that there is a dopaminergic hypofunction in RLS, this lack of decrease in Tyrosine Hydroxylase in substantia nigra is consistent with previously published post-mortem data in RLS. With Bonferroni correction, the decrease in thalamic Beta endorphin remained significant ($p=.006\times7=.042$). These results suggest that there may be altered central processing of pain in RLS and these data further implicate the endogenous opioid system in the pathogenesis of RLS. The mu opiate receptor subtype may be involved in the pathogenesis of RLS as it is the target of Beta-endorphin and Met-enkephalin but not Leu-enkephalin. However, these results should be viewed as only preliminary and more advanced techniques such as stereology should be employed in future post-mortem studies. In addition, other opioid rich areas need to be explored as well as areas implicated in the pathogenesis of RLS such as the red nucleus and raphe nucleus.

1. Introduction

Opioids effectively treat RLS as shown in a double-blind and long-term studies [1,2]. Opioid receptor PET scans show post-synaptic binding of ligand that is inversely proportional to the severity of RLS symptoms in areas serving the medial pain system including the thalamus [3]. With sensory discomfort, single photon emission computed tomographic (SPECT) scans showed reduced caudate nuclei activity, and increased thalamic and anterior cingulate activity in RLS patients, compared to controls [4]. A functional MRI study also implicates the thalamus during the sensory symptoms of RLS [5]. Furthermore, high resolution T-1 weighted MRI shows that RLS patients have increased gray matter in the thalamus [6]. If naloxone, an opioid antagonist, is given in a double-blind fashion to opioid treated RLS patients, the motor and sensory signs and symptoms return in a qualitative and quantitative fashion [7,8]. Additional receptor blocking studies indicate that opioids have their impact on RLS symptoms by modulating the dopamine system: Dopaminergic agonists are also therapeutic in RLS patients but naloxone only reverses the therapeutic effect of opioids whereas dopamine receptor blocking agents reverse the therapeutic effect of either the opioids or dopaminergic agonists [9]. There is possibly a mild functional dopamine deficiency, and a more profound iron deficiency in RLS patients at the nigro-striatal level [10,11]. In an in-vitro animal model of RLS, iron deprivation results in the death of dopaminergic cells in the substantia nigra and this cell death is prevented by the pre-administration of opioids to the cells [12]. In this animal model, dopamine cell death is presumed to be a surrogate for dopamine cell hypofunction given the fact that human autopsy data on RLS patients

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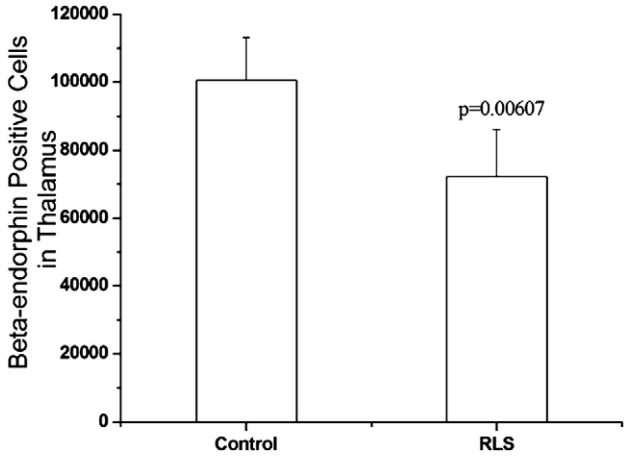

Fig. 1. Graphic representation of the difference in staining for Beta endorphin between RLS patients and controls. RLS patients had a 37.5% reduction (effect size 2.16) in staining for Beta-endorphin compared to controls (raw p value=.006, p value with Bonferroni correction=.042).

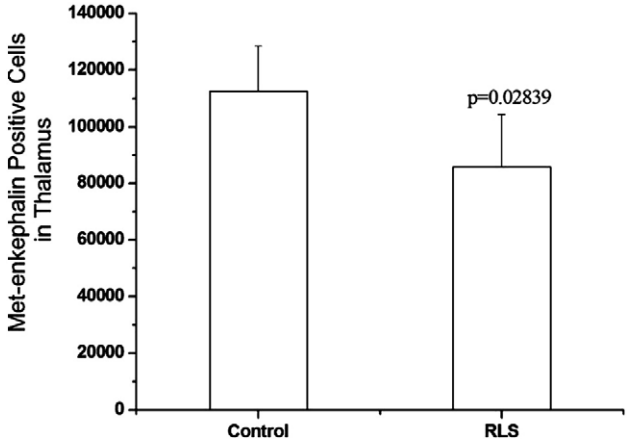

Fig. 3. Graphic representation of the difference in staining for Met-enkephalin between RLS patients and controls. RLS patients had a 26.4% reduction (effect size 1.58) in staining for Met-enkephalin compared to controls (raw p value=.028, p value with Bonferroni correction=NS).

does not show evidence of a dopamine deficiency [11]. All of these data suggest altered central processing of sensory information in RLS patients and implicate the endogenous opioid system in the sensory and motor aspects of RLS. The data also suggest an intimate relationship between iron, dopamine and opioids in the pathogenesis of RLS.

We therefore compared Beta endorphin, Met-enkephalin and Leu-enkephalin levels in the thalamus and substantia nigra of RLS patients compared to controls. Tyrosine Hydroxylase, the rate limiting step for dopamine synthesis was measured in the substantia nigra.

2. Methods

Brains from RLS patients (5 F — avg age 80.2 years) and controls (5 F, 1 M — avg age 76.3 years) were obtained from the Harvard Brain Bank and through the Restless Legs Syndrome Foundation.

2.1. Tissue collection

The brains were harvested at autopsy. One half of each brain was fixed in paraformaldehyde (PFA) in phosphate buffered saline (PBS) for pathologic evaluation. Cryostat sections were stained with antibodies Beta-endorphin (1:500, Chemicon, Temecula, CA, rabbit, polyclonal), Met-enkephalin (5ug/ml, Dako, Chemicon, Temecula, CA, rabbit, polyclonal), Leu-enkephalin (1:500, Chemicon, Temecula, CA, rabbit, polyclonal) and Tyrosine Hydroxylase (TH, 1:1500; Protos Biotech, New York, NY, rabbit polyclonal).

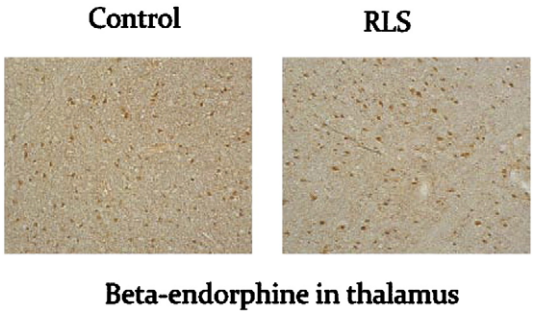

Fig. 2. Paraffin sections were stained with antibodies to Beta-endorphin (1:500, Chemicon, Temecula, CA, rabbit, polyclonal). RLS patients show less staining than controls.

Beta-endorphin, Met-enkephalin, and Leu-enkephalin were measured in both the substantia nigra and the thalamus. Since Tyrosine Hydroxylase is considered more relevant to dopaminergic nigro-striatal pathways, it was measured in the substantia nigra only.

Immunochemistry was performed on paraffin and cryostat sections cut at 30 μm thick sections. Sections were incubated with 0.3% H_2O_2 in PBS for 15 min at room temperature to block endogenous peroxidase. Nonspecific staining was blocked using 10% goat serum. Then the sections were incubated with primary antibodies diluted in 0.1 M PBS containing 1% BSA and 0.01% Triton X-100 at 4 °C overnight. The negative staining control slides were incubated with phosphate-buffered saline instead of primary antibody. In all cases, slides were incubated for 2 h at room temperature with the appropriate biotinylated secondary antibody. The avidin–biotin method was used to amplify the signal (ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA) and 3, 3′-diaminobenzidine tetrachloride (DAB) was used to visualize bound antibodies.

The slides of the thalamus and substantia nigra were studied at the fifth section with 150 μm intervals. A total 10 slides per patient were examined. Student t-tests were used to compare patients and controls and a Bonferroni statistical correction was applied to the final results. As the slides were de-identified by name and diagnosis, cell counts were done in a blinded fashion.

3. Results

In the thalamus, there were statistically significant reductions of Beta-endorphin cells by 37.5% (p=.006) and Met-enkephalin positive cells by 26.4% (p=.028), in RLS patients compared to controls (Figs. 1–4). The effect sizes for these changes were also quite large at 2.16 and 1.58, respectively. However, the number of Leu-enkephalin positive cells in thalamus of RLS patients remained the same as that in controls. In the substantia nigra, the ß-endorphin, Met-enkephalin and Leu-enkephalin immunoreactive cells shared the same distribution as Tyrosine Hydroxylase positive cells. There were no significant differences in the immunoreactivities of these four antibodies in the substantia nigra in RLS patients compared to controls (Figs. 1–4). With Bonferroni correction of the significant p values, the decrease in thalamic Beta endorphin remained significant at p=.006×7=.042.

Time from death to freezing of the brains did not differ significantly between the patients (18.4 h) and the controls (17.2 h) and there was no correlation between the numbers of cells and the time from death to freezing for either Beta endorphin or Met-enkephalin. We obtained