Dreaming permits each and every one of us to be quietly and safely insane every night of our lives.

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The results are summarized under the following headings:

5.1 Effect of REM sleep deprivation on cell size

i) In Nissl stained sections.

ii) In TH, ChAT and GAD immunostained sections.

5.2 Effect of REM sleep deprivation on intensity of color in immunostained sections.

5.3 Effect of REM sleep deprivation on neuronal degeneration

i) In Amino cupric silver stained sections

ii) In bcl2 and bax stained sections

iii) In TUNEL stained sections

iv) In electron micrographs

5.4 Effect of REM sleep deprivation on structural proteins (in actin stained sections).

5.1 EFFECT OF REM SLEEP DEPRIVATION ON CELL SIZE:

In this section, mean cell perimeter and mean area after REM sleep deprivation (REMSD) was compared with that of the respective mean values in Free Moving Control (FMC), Large Platform Control (LPC) (done to study the effect of non-specific stress), recovery (REC) (to study the recovery from REMSD) and after injection of prazosin. All the data have been represented in 3 ways: as graphs comparing means of actual values (group A’s), as graphs comparing percentage mean values (group B’s) and as frequency distribution histograms representing the number of neurons in each cell size class.

*Frequency distribution histogram:* This type of histogram represents the data as a correlation between the sizes of cells and the cell frequency. The cell sizes are grouped into bins with a specific range on the X-axis and the number of cells in each bin is represented on the Y-Axis. This method of representation enables segregation of cells into groups according to their sizes. This also gives a graphic picture of the number of cells per
group. In the present study, the bins were of a difference of 20 μm for perimeter and 50 μm² for area. The frequency histograms were also represented as bars graphs (A’s) and line graphs (B’s). The actual values for perimeter and area were represented as tables.

5.1.1 Nissl stained neurons

(a) Locus Coeruleus (LC):

In LC, the mean perimeter of the neurons increased to 114.03% after REMSD as compared to FMC taken as 100% \([F(1,S)=91.8, p<0.001]\) (Fig 1A,B; Table 1). The frequency distribution histograms also indicated that there was a shift towards larger cell size class after REMSD (Fig 2A,B). Similarly, the area increased to 119.8% in REMSD \([F(1,S)=91.8, p<0.001]\) (Fig 3A,B; Table 1) which was also observed in the frequency distribution histogram (Fig 4A,B). In LPC, both the perimeter and area remained similar to FMC. After REC and prazosin injection (PRZ) the increased perimeter and area returned to FMC values.

(b) Laterodorsal tegmentum and pedunculopontine tegmentum (LDT/PPT):

In LDT/PPT, the perimeter of the Nissl stained neurons decreased to 85.15% after REMSD as compared to FMC \([F(1,5)=79.8, p<0.001]\) (Fig 5A,B; Table 2). The frequency distribution histograms also showed that more neurons after REMSD were present in the lower perimeter class (Fig 6A,B). The area decreased to 82.38% after REMSD \([F(1,5)=249.6, p<0.001]\) (Fig 7A,B; Table 2). The frequency distribution histogram represented in Fig 8 (A, B) also shows a shift towards the lower cell area class after REMSD. The corresponding values in LPC, REC and PRZ for both perimeter and area remained similar to FMC.
(c) **medial Preoptic Area (mPOA)**:

In the mPOA, perimeter decreased to 78.56% after REMSD compared to FMC [F(1,5)=73.0, p<0.001] (Fig 9A,B; Table 3). Similar observation was made in the frequency distribution histogram (Fig 10A,B). Similarly, the area decreased to 78.46% after REMSD [F(1,5)=144.2, p<0.001] (Fig 11A,B; Table 3) which was also represented in the frequency distribution (Fig 12A,B). Both the perimeter and area in LPC, REC and PRZ were similar to the corresponding FMC values.

(d) **Lateral septum**:

Interestingly in the lateral septum no significant change was observed in either the perimeter or the area of neurons after REM sleep deprivation. All the values remained nearly equal in all the five groups for perimeter (Fig 13A,B; Table 4) and area (Fig 15A,B; Table 4). A similar trend was observed in the corresponding frequency histograms (Fig 14A,B; Fig 16A,B).

Since no change was observed in the size of neurons in the lateral septum in the nissl stained sections, this area was not considered for immunohistochemistry. Moreover, previous studies had reported that this area lacked both ChAT and GAD positive neurons (Gritti et al., 1993). Hence in the present study, TH staining was done in LC (where noradrenergic neurons have been reported, ChAT in LDT/PPT (which has cholinergic neurons) and GAD was done in LC, LDT/PPT and mPOA.

5.1.2 **Immunostained neurons**:

(a) **TH stained neurons in LC**:

After staining with antibody against tyrosine hydroxylase (TH), the perimeter increased to 116.16% [F(1,5)=37.7, p<0.01] (Fig 17A,B; Table 5) while area increased to
116.76% [F(1,5)=80.4, p< 0.001] (Fig 19A,B; Table 5) after REMSD as compared to FMC. The LPC and REC values were similar to FMC. A shift to larger cell size bin was also observed in the frequency distribution for perimeter (Fig 18A,B) and area (Fig 20A,B).

Representative TH stained neurons in FMC, REMSD, LPC and REC groups are shown in Photo1.

(b) **ChAT stained neurons in LDT/PPT:**

In the neurons stained with antibody against ChAT enzyme the neuronal perimeter decreased significantly to 85.45% after REMSD [F(1,5)=81.6, p< 0.001] (Fig 21A,B; Table 6) while area significantly decreased to 82.2% as compared to FMC [F(1,5)=184.4, p< 0.001] (Fig 23A,B; Table 6). LPC and REC values remained unchanged compared to FMC. A shift towards the lesser cell size class was observed in the frequency distributions for perimeter (Fig 22A,B) and area (Fig 24A,B).

Representative ChAT stained neurons from FMC, REMSD, LPC and REC groups under different magnifications are shown in Photo2.

(c) **GAD stained neurons:**

i) **In LC:**

After staining with GAD antibody in LC, the perimeter of GAD positive neurons increased to 103.96% after REMSD as compared to FMC [F(1,5)=8.38, p< 0.05] (Fig 25A,B; Table 7). Similarly, area of GAD positive neurons increased to 109.7% as compared to FMC [F(1,5)=40.2, p< 0.01] (Fig 27A,B; Table 8). The LPC and REC values were not significantly different from FMC. The frequency distribution histograms for both
perimeter (Fig 26A,B) and area (Fig 28A,B) showed that there were more number of neurons in the larger cell size bins after REMSD as compared to FMC.

**ii) LDT/PPT:**

In the neurons stained for antibody against GAD the perimeter increased to 113.7% after REMSD \( [F(1,5)=26.0, p<0.01] \) (Fig 29A,B; Table 8) while area increased to 120.5% as compared to FMC \( [F(1,5)=27.6, p<0.01] \) (Fig 31A,B; Table 8). For LPC and REC the corresponding values were not significantly different from FMC. A similar pattern of distribution was also observed in the frequency histograms for perimeter (Fig 30A,B) and area (Fig 32A,B).

**iii) mPOA:**

After staining with antibody against GAD in mPOA, the perimeter of neurons decreased to 85.06% after REMSD \( [F(1,5)=64.5, p<0.01] \) (Fig 33A,B; Table 9) while the area decreased to 81.1% after REMSD as compared to FMC \( [F(1,5)=46.2, p<0.01] \) (Fig 35A,B; Table 9). The corresponding values for LPC and REC were similar to FMC. A similar distribution was observed in the frequency distribution histograms for perimeter (Fig 34A,B) and area (Fig 36A,B).

GAD stained neurons in different magnifications in FMC, REMSD, LPC and REC groups are shown in Photo3.

5.2 **EFFECT OF REM SLEEP DEPRIVATION ON DENSITOMETRIC ESTIMATION OF INTENSITY OF COLOR IN IMMUNOSTAINED SECTIONS.**

**(a) Effect on TH stained neurons in LC:**

The intensities of color, as obtained by the densitometric evaluation of the immunostained enzymes, were directly proportional to the concentration of the enzyme in
the neuron. The color intensity of TH per unit area significantly increased to 153.9% after REMSD as compared to FMC \( [F(1,5) = 252.6; p<0.001] \) (Fig 37A,B; Table 10). The color intensities of the TH enzyme in LPC and REC groups of rats were comparable to that of FMC.

\[ (b) \textit{Effect on ChAT stained neurons in LDT/PPT :} \]

The intensity per unit area of ChAT slightly decreased in the LDT/PPT from 100% in FMC to 91.0% in REMSD, however, it was not statistically significant. The values in LPC and REC groups were comparable to that of the FMC (Fig 38A,B; Table 11).

\[ (c) \textit{Effect on GAD stained neurons} \]

\[ i) \textit{In LC :} \]

The color intensity of GAD in LC significantly \( [F(1,5) = 79.4; p<0.001] \) increased to 133.3% after REMSD as compared to FMC (Fig 39A,B; Table 12). The intensities in LPC and REC groups were not significantly different from FMC.

\[ ii) \textit{In LDT/PPT :} \]

There was slight increase in GAD intensity in the LDT/PPT as well, however, the increase was statistically non-significant. The values increased to 111.1% after REMSD as compared to FMC while the values in LPC and REC groups were comparable to that of the FMC (Fig 40A,B).

\[ iii) \textit{In mPOA :} \]

The intensity of GAD per unit area of neurons in mPOA non-significantly decreased to 93.9% after REMSD as compared to FMC. The values of LPC and REC were also not different from FMC (Fig 41A,B).
5.3 EFFECT OF REM SLEEP DEPRIVATION ON NEURONAL DAMAGE:

In this section, the data was represented as mean percentage of degenerated cells in LC, LDT/PPT and mPOA in REMSD and control brains. Initially a comparison was made between the percentage of amino-cupric-silver stained degenerated neurons in FMC and that in 4, 6 and 10 days of REMSD. It was observed that after 6 and 10 days REMSD, a significant percentage of neurons were found to be degenerated as compared to FMC in all three areas. However, after 4 days of REMSD, the percentage of degenerated neurons did not significantly increase as compared to FMC. Since, it was not desirable to subject the rat to unwanted deprivation but at the same time to subject them to minimum deprivation to get significant effect on the parameter to study, it was decided to do the further experiments after 6 days of REM sleep deprivation. Therefore the large platform control and recovery studies were done along with the 6 days deprivation studies and the results are reported later. In this section the results of 4 days and 10 days of REMSD in different areas have been reported.

In LC:

The mean percentage of degenerated cells in LC increased significantly after 10 days REMSD as compared to FMC \([F(1,5) = 489.7; p<0.001]\). After 4 days of REMSD the increase in the percentage of degenerated neurons was not significant compared to FMC (Fig 42A,B; Table 15).

In LDT/PPT:

The trend was similar in LDT/PPT to that in LC where a significant percentage \([F(1,5) = 337.0; p<0.001]\) of neurons was found to be degenerated after 10 days of REMSD compared to FMC. Neuronal degeneration after 4 days REMSD was not significantly different from FMC (Fig 42A,B; Table 15).
**Results**

*In mPOA:*

Similarly, a significant percentage of neuronal degeneration \([F(1,5) = 132.3; p<0.001]\) was observed after 10 days REMSD in mPOA as compared to FMC. The percentage of degeneration neurons after 4 days of REMSD was not significant (Fig 44A,B; Table 15).

*In lateral septum:*

There was no significant difference between percentage of degenerated cells in FMC and 4, 6 or 10 days of REMSD in the lateral septum (Fig 45A,B; Table 15).

Since it was reported that the cell size is altered when neurons undergo degeneration (Wyllie et al., 1980), studies were undertaken in which neuronal degeneration was studied first using electron microscopy and subsequently using other protocols that stain degenerated neurons.

**Transmission Electron Microscopy:**

Following transmission electron microscopy degenerative changes were visible in all three areas (LC, LDT/PPT and mPOA) after REM sleep deprivation. The majority of neurons after REMSD showed increased chromatin condensation along the nuclear membrane, clumping of chromatin material inside the nucleus, a general disorganization of the cell organelles and subsequent nuclear lysis that are all hallmarks of apoptosis. FMC and LPC had neurons that were normal looking with clear nuclear membrane, presence of nucleolus and well defined cell organelles. Recovery had both normal and apoptotic neurons indicating a partial recovery from the effects of deprivation.

Electron micrographs of neurons in LC, LDT/PPT and mPOA after FMC, REMSD, LPC and REC are shown in Photo 14, 15 and 16 respectively. For comparison between the areas, the photographs are combined as a single figure in Photo 17.
Neuronal damage observed after Amino Cupric Silver staining:

In electron micrographs, the neurons from the brains areas after REM sleep deprivation showed the presence of degenerated neurons. To study this further, the brain areas were stained with the amino-cupric-silver technique. This technique is based on the fact that degenerating neurons have increased affinity for binding with silver or to become argyrophillic. Moreover it stains neurons that are still undergoing degeneration, hence some of the neurons that might recover back can also be stained with this technique.

*In LC:*

In the LC the mean percentage of degenerated neurons significantly increased from 17.13 ± 0.44 % in FMC to 52.4 ± 2.31 % after REMSD [F(1,5)=225.0, p< 0.001]. In LPC, 19.73 ± 4.71 % of the neurons were degenerated. The percentage of degenerated cells in REC 23.13 ± 2.21 % was not significantly different from FMC (Fig 46; Table 16).

Amino-cupric-silver stained neurons in LC after FMC, REMSD, LPC and REC are shown in Photo4.

*In LDT/PPT:*

In LDT/PPT the percentage of degenerated neurons significantly increased from 19.16 ± 1.16 % in FMC to 52 ± 1.80 % in REMSD [F(1,5)=235.2, p< 0.001]. Both the LPC value of 19.16 ± 1.70 % and the recovery values of 25.9 ± 2.67 % were not significantly different from FMC (Fig 47; Table 16).

Amino-cupric-silver stained neurons in LDT/PPT after FMC, REMSD, LPC and REC are shown in Photo5.
Results

**In mPOA:**

In mPOA, the degenerated neurons significantly increased in percentage from 15.96 ± 0.29 % in FMC to 50.23 ± 3.20 % in REMSD \( [F(1,5)=113.8, p<0.001] \). The LPC value of 17.26 ± 0.73 % was similar to that of FMC. The neurons recovered after REM sleep deprivation as a non-significant percentage 26.8 ± 5.6 % was found to be degenerated after recovery (Fig 48; Table 16).

Amino-cupric-silver stained neurons in mPOA after FMC, REMSD, LPC and REC are shown in Photo6.

**In lateral septum:**

In the lateral septum there was no change in the percentage of degenerated neurons which remained between 15% and 16% in all four groups (FMC-15.03 ± 0.24 %, REMSD-15.43 ± 0.52%, LPC-15.35 ± 0.15 %, REC-15.80 ± 0.60%) (Fig 49; Table 16).

Amino-cupric-silver stained neurons in lateral septum after FMC, REMSD, LPC and REC are shown in Photo7. In Photo 8 photographs of all the four areas (reduced in size) have been shown together for comparison between the different areas.

Thus, it was observed that in the areas LC, LDT/PPT and mPOA there was increased percentage of degenerated neurons after REMSD as compared to FMC. However, amino-cupric-silver technique stains those neurons that are undergoing degeneration but still have the capacity to recover. Further, it does not distinguish whether the type of degeneration is apoptosis or necrosis. Hence, the later experiments by immunostaining with bcl-2 and bax antibodies and TUNEL were performed to study the incidence of apoptosis in these neurons. Since no change was observed in lateral septum, further experiments were not done in this area.
Percentage of bcl-2 and bax positive neurons:

Bcl-2 is an anti-apoptotic protein that is present on the mitochondrial and nuclear membranes and prevents the activation of caspases and the apoptotic process. Bax is a pro-apoptotic protein that is expressed during apoptosis. The relative percentage of bcl-2 and bax expressing neurons give an indication whether the cells have an increased incidence of apoptosis.

In LC:

In LC the mean percentage of bcl-2 positive neurons was found to be maximum in FMC (77.03 ± 3.89 %) while the corresponding bax positive neurons were minimum in FMC (22.9 ± 3.91%). The percentage of bcl-2 positive neurons was least in REMSD (41.53 ± 5.34 %) that significantly differed from FMC [F(1,5)=28.9, p< 0.01] (Fig 50A; Table 17) The REMSD group also showed a high percentage of bax positive neurons (58.40 ± 5.37%) which was significantly different from FMC [F(1,5)=28.6, p< 0.01] (Fig 50B; Table 18). In LPC and REC, both bcl-2 and bax percentages were not significantly different from their respective FMC values.

Bcl-2 and bax stained neurons in LC after FMC, REMSD, LPC and REC are shown in Photo9.

In LDT/PPT:

In LDT/PPT the percentage of bcl-2 positive neurons in FMC was 77.55 ± 3.97 % and bax positive neurons were 22.36 ± 3.95 %. REMSD had a low bcl-2 percentage (43.53 ± 0.5 %) which was significantly different from FMC [F(1,5)=72.3, p< 0.001] (Fig 51A; Table 17). Percentage of bax positive cells was high in REMSD (56.40 ± 0.47 %) and significantly different from bax percentage in FMC [F(1,5)=73.2, p< 0.001] (Fig 51B;
Table 18). The relative bcl-2 and bax percentages in LPC and REC were not significantly different from FMC.

**In mPOA:**

In mPOA bcl-2 mean percentage was again high in FMC (76.03 ± 2.97%) and bax low (23.90 ± 2.97%). In REMSD percentage of bcl-2 positive neurons (45.86 ± 1.18 %) were less than the percentage of bax positive neurons (54.06 ± 1.21%). In both case the bcl-2 and bax percentage were significantly different from their respective FMC values; [F(1,5)=89.1, p< 0.001] for bcl-2 (Fig 52A; Table 17) and [F(1,5)=88.4, p< 0.001] (Fig 52B; Table 18). In FMC and REC both percentage of bcl-2 and bax positive cells were not significantly different from their respective FMC values.

Thus, it was observed that in all three areas the percentage of bcl-2 stained neurons was lower and the percentage of bax stained neurons was higher after REMSD as compared to FMC. Bcl-2 is an anti-apoptotic protein while bax is pro-apoptotic. Hence, the results show that there is increased expression of pro-apoptotic proteins after REMSD while the expression of the proteins that block apoptosis is decreased. This indicates that after REMSD, there is an increased tendency of the neurons to go into the apoptotic pathway. The percentage of cells that actually undergo apoptosis was further studied in the following experiments using the TUNEL technique.

**Percentage of TUNEL positive neurons:**

The TUNEL technique labels the DNA nicks in the nuclei of apoptotic cells. Thus it preferentially labels apoptotic cells and does not label cells that die of other forms of degeneration. Hence it is specific for detection of apoptosis in a system.
**In LC:**

In the LC the mean percentage of TUNEL positive neurons significantly increased from $11.0 \pm 3.09\%$ in FMC to $43.23 \pm 3.78\%$ after REMSD $[F(1,5)=43.6, p<0.01]$ (Fig 53; Table 19). In LPC and REC the percentage of neurons stained for TUNEL was not significantly different than FMC.

TUNEL stained neurons in LC after FMC, REMSD, LPC and REC are shown in Photo10.

**In LDT/PPT:**

In LDT/PPT the percentage of TUNEL positive neurons significantly increased from $11.31 \pm 1.13\%$ in FMC to $45.94 \pm 2.34\%$ in REMSD $[F(1,5)=176.3, p<0.001]$. The LPC value was not significantly different from FMC. However after recovery, a significant percentage $[F(1,5)=18.3, p<0.05]$ were found to be TUNEL positive though the value was less than in REMSD (Fig 54; Table 19).

TUNEL stained neurons in LDT/PPT after FMC, REMSD, LPC and REC are shown in Photo11.

**In mPOA:**

In mPOA, the TUNEL positive neurons significantly increased from $9.83 \pm 2.008\%$ in FMC to $47.0 \pm 2.73\%$ in REMSD $[F(1,5)=120.5, p<0.001]$. The LPC value was comparable to that of FMC. The neurons recovered partially after REM sleep deprivation though a significant percentage $[F(1,5)=29.3, p<0.05]$ were found to be TUNEL positive (Fig 55; Table 19). However, this percentage was less than REMSD, indicating that the neurons had partially recovered.
TUNEL stained neurons in mPOA after FMC, REMSD, LPC and REC are shown in Photo12. In Photo 13, the photographs of all 3 areas have been shown together for convenience of comparison.

5.4 EFFECT OF REM SLEEP DEPRIVATION ON STRUCTURAL PROTEINS:

Densitometric estimation of the staining intensity was done after immunostaining with the antibody against the structural protein actin. The intensity of color, as obtained by the densitometric estimation of the immunostained sections in different brain areas, was directly proportional to the number of protein molecules per unit area of the neuron.

In LC:

After REMSD, the mean density of color per unit area decreased significantly in LC to 63.66%, the FMC being 100% [F (1,5)=62.5, p<0.001] (Fig 56A,B; Table 20). The color intensities in LPC and REC groups were comparable to that of the FMC.

In LDT/PPT:

In LDT/PPT, the mean density of color per unit area decreased significantly after REMSD to 70.23% as compared to FMC taken as 100% [F (1,5)=36.1, p<0.01] (Fig 57A,B; Table 20). The values in LPC and REC groups were comparable to that of FMC.

In mPOA:

The mean density of color per unit area in mPOA decreased significantly after REMSD to 76.56% as compared to FMC taken as 100% [F (1,5)=2405, p<0.01] (Fig 58A,B; Table 20). The values in LPC and REC groups were comparable to that of FMC.
The results from this study may be summarized as follows:

1. REM sleep deprivation affected the size of neurons located in the areas involved in REM sleep regulation. After nissl staining, both the perimeter and area of neurons in LC increased while those parameters decreased in LDT/PPT and mPOA following REM sleep deprivation. No change was observed in the lateral septum. The alterations returned to control levels after recovery and after prazosin injection.

2. The perimeter and area of TH positive (noradrenergic) neurons in LC increased while that of ChAT positive (cholinergic) neurons in LDT/PPT decreased after REM sleep deprivation. Among the GAD positive (GABAergic) neurons, those parameters increased in LC and LDT/PPT while they showed a decrease in mPOA. The changes returned to control levels after recovery.

3. After REM sleep deprivation densitometrically estimated TH and GAD enzyme concentrations increased only in LC while there were no significant changes in the ChAT and GAD enzyme concentrations in LDT/PPT and mPOA.

4. Alterations in cellular morphology could be the initial expression for neuronal degeneration due to apoptosis. The ultrastructure of the neurons in LC, LDT/PPT and mPOA after REM sleep deprivation and control were examined by electron microscopy. The electron micrographs showed that in the REM sleep deprived samples there was intense chromatin condensation in the nuclei and marginalization of the chromatin along the nuclear membrane indicating that the neurons might have undergone apoptotic changes.

5. To further confirm neuronal degeneration after REM sleep deprivation, amino-cupric silver staining was done. The percentage of amino-cupric-silver stained degenerated neurons was found to increase in LC, LDT/PPT and mPOA after REM
sleep deprivation. No significant increase in number of degenerated neurons was observed in the lateral septum.

6. The percentage of neurons expressing bcl-2 (anti-apoptotic protein) were significantly decreased while bax (pro-apoptotic protein) increased after REM sleep deprivation in LC, LDT/PPT and mPOA.

7. The percentage of TUNEL positive (apoptotic) neurons increased after REM sleep deprivation in LC, LDT/PPT and mPOA.

8. Since alterations in size of cells may be due to changes in the structural proteins and the structural proteins are affected in apoptosis, it was attempted to estimate the concentration of the structural protein, actin. It was observed that after REM sleep deprivation there was a decrease in actin concentration in all three areas studied viz. LC, LDT/PPT and mPOA.