Olfactory tract transection in neonatal rats: Evidence for Mitral cell regeneration and restoration of functional connectivity with its targets

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Central Nervous System (CNS) regeneration and repair mechanism are two important aspects of functional recovery in the adult central nervous system following brain and spinal cord injury. Following olfactory tract transection in neonatal rats, functional connectivity between the olfactory bulb and the piriform cortex gets re-established by 120 days. The recovery of the dendritic morphology was associated with the synchronized oscillatory activity between olfactory bulb and piriform cortex. Mitral cells which were regenerated after the transection showed profuse branching, indicative of their undifferentiated state. However, normal dendritic morphology could be seen by 120 days after olfactory tract transection. These results thus provide a supportive evidence for the restoration of the functional connections between the olfactory bulb and the piriform cortex at 120 days.

Keywords: Local field potentials, Mitral cells, Olfactory tract transection, Piriform cortex, Regeneration

The mammalian olfactory system has a remarkable capacity to continuously form new interneurons in adulthood under normal conditions as well as following an insult. The importance of host regeneration as the possible mechanism leading to the functional recovery following nerve transection has been reported. Munirathinam et al., have demonstrated the axonal regeneration following olfactory tract transection. However, the study did not provide any evidence for the functional restoration in terms of integrated electrical activity between the bulb and the piriform cortex following olfactory tract transection.

The Mitral/tufted cells of the olfactory bulb (OB) are extensively connected with piriform cortex and various higher cortical regions of the brain. Owing to these extensive connections with both neocortical and subcortical structures, including the hippocampus, olfactory system is involved in multiple behaviours such as detection of odours, food-seeking behaviour, spatial memory and various maternal behaviours.

The olfactory system undergoes both functional and anatomical changes during the first few weeks of postnatal life. Olfactory tract transection (OTT) at this stage causes a wide range of functional and cytoarchitectural changes. However, the tract is capable of undergoing regeneration and thus sparing the behavioural abnormality as shown in male hamsters. The reinnervation of the olfactory bulb with its target is specific to the regenerative capability of Mitral cells of the olfactory bulb which is aided by the olfactory stimulation. After 45 days post-transection, the olfactory tract fibers start regenerating and by 240 days the fiber density was near to normal. It perhaps could be due to the unique properties of neural progenitor cells of the subventricular zone which migrate to the olfactory bulb where they differentiate into interneurons, like granule and periglomerular cells in adults. The migrating subventricular cells are capable of forming Mitral cells in the transected rats.

One of the effective strategies to probe how the new connections are formed during regenerative process is through investigating the morphological changes in the Mitral cells following OTT. Analysis of local field potentials (LFPs) combined with dendritic morphology of Mitral cells could substantiate the establishment of functional connectivity. This is important because synchronized...
oscillatory activity at theta frequency (4-12Hz) is thought to be important for information processing in any sensory systems\textsuperscript{30-36}.

Accordingly, the present study reports a detailed morphological assessment of regenerating Mitral cells following OTT in Albino rat pups. The restoration of functional integrity between the olfactory bulb and its targets has been evaluated electrophysiologically by studying the LFPs from olfactory bulb and piriform cortex.

**Materials and Methods**

**Animals**—Albino rat pups (P0) of Wistar strain were bred and raised in the Central Animal Research Facility (CARF) of the Institute. Rats housed in polypropylene cages were maintained on a 12 hour light/dark cycle and given food and water *ad libitum*. All the experimental work was carried out with due clearance from the Institutional animal ethics committee. It was ensured that the room was properly ventilated and the ambient temperature was maintained.

**Experimental groups**—A total 144 male rats were used. All rat pups were randomly divided into three groups, lateral olfactory tract transected (OTT), sham control (Sham) and normal control (NC). OTT was achieved by lateral olfactory tract (LOT) transection at postnatal day 4 (P4). Sham animals received the same treatment, except that the LOT was not transected. Normal control animals were not exposed to any surgical procedures.

**Surgery**—Olfactory tract transection procedure was carried out under halothane anesthesia as reported earlier with a few modifications\textsuperscript{33,37}. The olfactory tract was accessed by unilateral incision on the skull, posterior to the olfactory bulb. A sterile surgical blade (no.11) was inserted along the orbital surface of frontal bones and gently drawn back and forth to ensure complete bilateral transection of the tract. The wound was cleaned with antiseptic and sealed with Healex spray. Following surgery the pups were warmed and returned to their mothers or colony. Same day old littermates were used as experimental controls.

**Tissue preparation for the morphological studies**—After completion of experimental procedures, rats (n=8-10 per group) from all groups (NC, SC and OTT) were sacrificed by decapitation at two time points: 70 and 120 days following transection. The cerebrum and olfactory bulbs were removed together, carefully avoiding any damage to the bulbs. The tissues including the frontal cortex were processed for rapid Golgi impregnation\textsuperscript{38-40}. After impregnation, the olfactory bulbs were carefully removed and embedded in hot wax. The sagittal sections of 140-160 µ thickness were obtained using rotary microtome. Camera lucida neuronal tracing of Mitral cells (with minimal damage, high quality of impregnation) was done under 625x magnification using a Leica microscope (Leica, Germany). The criteria for selection of Mitral cells for drawing were: (i) neurons must be confined to the Mitral Cell Layer, (ii) they must be darkly stained, and (iii) neuron should not have any truncation of branches. Neurons were chosen from identical regions of olfactory bulbs. A total 60-80 neurons (Mitral cells per group) were used for quantitative analysis of dendritic morphology.

To study the changes in the dendritic arborization, the dendritic intersections of the Mitral cells were taken with 20 µ increment using Sholl’s analysis\textsuperscript{41}. In brief, concentric circles were drawn in a transparent sheet of 20 µ increments using a stage micrometer scale at the same magnification at which the neurons were drawn. Using the center of the cell as a reference point, intersections and branching points were measured from the cell body in each successive circle. The number of dendritic branching points within two adjacent concentric circles was counted as described earlier\textsuperscript{38-40}. The dendritic branching points and intersections were studied up to a distance of 260 µm from the soma.

**Local field potential (LFP) recordings**—A total 35 rat pups (n=8-10 per group) were used for the electrophysiological study. The rats were randomly divided into following four groups; normal control (NC) for 70 days (n=8), NC for 120 days (n=7), OTT for 70 days (n=10) and OTT for 120 days (n=10).

**Implantation procedure**—Rats were anesthetized with a combination of Ketamine (80 mg/kg body weight) and Xylazine (10 mg/kg body weight). In addition, xylocaine (2%) was injected subcutaneously before the surgery. The depth electrodes were carefully implanted stereotaxically on bilateral sides into the olfactory bulb and Piriform Cortex (PCx) as per the dorso-ventral (DV) co-ordinates adapted from Paxinos and Watson\textsuperscript{42}. The insulated electrodes were implanted stereotaxically into the olfactory bulb (Mitral cell layer (MCL) (anterior-posterior (AP): +6.7 mm, medio-lateral (ML): 2.0 mm, dorsoventral (DV): 2.5 mm) and layer 3 PCx (AP: +1.5 mm, ML: +1.0 mm, DV: 0.8 mm).
Grounding electrode was placed in the right parietal cortex. All these electrodes were soldered and assembled into a 5-pin integrated circuit socket and the connector was fixed to the skull using the dental acrylic cement (Dental Products of India Ltd., Mumbai, India) and was allowed to recover from surgery. Healex was applied to all the wound edges and the rat was finally placed back in its home cage for 5 days for the recovery from the surgical effects. Following surgical recovery, LFPS recordings were carried out in their familiar home cage concurrent with the spontaneous behaviour.

After 4-6 days of post surgical recovery, the rats were connected to the 8-channel LFPS Polygraph (Grass Model 78D Polygraph) via a flexible, shielded cable, which did not restrain or cause discomfort to the animal. The rat was provided with food and water ad libitum during the recording session. The rats were kept in the home cage with the electrodes connected to the socket for the exploration of the environment, prior to the recording session. The recording session lasted for 10 min and the spontaneous behaviour of the rat such as exploratory locomotion, grooming, rearing and in the stationary phase (quiet) was monitored throughout the recording session and marked.

**Offline analysis**—All the spontaneous behaviours exhibited by the adult transected and normal control rats were quantified offline using spike 2 software. The total percentage of different spontaneous behaviour was taken. Spontaneous changes in the behavioural pattern of rats in their home cage following OTT and behavioural recovery of rats after 70 and 120 days post-transection were compared with the data obtained from normal control rats. The changes in behavioural patterns correlated with the Mitral cell regeneration in rats. The analysis was done in both normal control and olfactory tract transected rats. For each time point, data from 10 rats per group were used for both NC and OTT rats.

**Results**

**Body weight changes due to OTT**—The body weight was evaluated every week after surgery and the increase in weight was compared with that of control rats. The mean body weight gain of OTT rats did not differ significantly from control rats (Fig. 1).

**Morphology of the olfactory bulb**—Rapid Golgi silver impregnation staining techniques were employed in all groups of rats to assess the cytoarchitecture of Mitral cells as well as structural changes in the dendritic arborization due to OTT. The olfactory tract appeared to establish an anatomical continuity with their target by 70 days post transection. Gradual recovery in the size of olfactory bulb in 70 days post transection and with a complete recovery in the bulb size by 120 days was also observed.

**Morphological assessment of dendritic arborization**—A representative example of camera lucida tracings and photomicrographs of rapid Golgi
stained olfactory bulb neurons from NC and OTT are given in Figs. 2a and b as well as 3a and b.

To investigate the impact of OTT on dendritic arborization in a greater detail, a segmental analysis was performed using the two-way ANOVA to track the changes in dendritic length and branch points as a function of radial distance from the cell soma. The statistical analysis suggest that OTT induces significant changes in the dendritic arborization of Mitral cells with a significant increase in the total number of dendritic branches ($F_{22,248}=2.263, P<0.001$) and dendritic intersection ($F_{18,208}=3.782, P<0.0001$). After 70 days of OTT, there was a pronounced decrease in the number of branching points of Mitral-neurons within a distance of 120-160 µm from the soma as revealed by Bonferroni’s post-hoc test (Fig. 2c). In addition, an increased number of branches within a distance of 40-80 µm from the soma were observed in 70 days of the OTT group (Fig. 2d). However, the numbers were comparable to that of control rats by 120 days ($F_{18,210}=1.559, P<0.07$) (Fig. 3d). Bonferroni’s multiple comparison test further revealed that dendritic hypertrophy induced by OTT was gradually ameliorated from 70 days (Fig. 2c) to 120 days (Fig. 3c) after OTT with a significant reduction in the number of intersections at 100-140 µ segment from the soma respectively. The differences in both dendritic intersections and in dendritic branches were not prominent between the control and sham control groups at any time points (Figs 2 and 3).

In addition to dendritic intersections and dendritic branch points, we have also observed several structural differences in Mitral cells between NC and OTT rats. Firstly, the olfactory tract transection induced distortion in the Mitral cell layer (MCL) of the 70 days OTT group (Fig. 4). Secondly, non-spiny Mitre-shaped cells were also observed in the central core of the olfactory bulb near to the rostral migratory stream (RMS). Thirdly, compared with normal olfactory bulb, olfactory bulb of OTT group showed increased vascularity characteristic feature of regenerating tissue during establishment of its connections with its target. Finally, more studded spinier granule cells were specifically detected in the granule cell layer of OTT rats indicating neural reaction to olfactory tract transection.

**Histological confirmation of the electrode placement**—Histological analysis with Cresyl violet stained sections confirmed that all bipolar

![Fig. 2—Olfactory tract transection after 70 days increases dendritic arborization in short shaft Mitral neurons of olfactory bulb compared with NC and Sham. (A) representative Mitral cell tracing from 70 days OTT group; (B) example of Golgi stained Mitral cell; (C) dendritic intersections; (D) total number of dendritic branches of Mitral cells at various distance from soma showing nerve sprouting following 70 days post-transection (n=10/group and 10 neurons/rat). NC=normal control, Sham=sham control, OTT=olfactory tract transected. Values are mean ± SE. P values: ** P value: <0.01, *** P value: <0.001 between NC and OTT rats.]
electrodes were indeed implanted into the MCL and PCx (layer 3) (Fig. 5).

Spontaneous exploratory behaviour of the rats in home cage—The home cage behaviour of the rat was compared between intact (NC and sham) and rats with bilateral OTT. After 70 days of olfactory tract transection, OTT rats reared more frequently than NC rats ($P<0.001$) (Fig. 6). However, 120 days OTT rats exhibited increased exploratory locomotion (t-test, $P<0.05$) and with normal spontaneous behaviours.

Associative changes in the neuronal activity of MCL and PCx during spontaneous exploratory locomotion behaviour in the home cage—Since the exploratory locomotor activity in the home cage has
to be considered in evaluating motivation-induced responses following transection, a detailed spectral analysis of the neuronal activities recorded from olfactory bulb and Piriform Cortex during this behaviour was carried out.

A typical example showing the local field potentials recorded bilaterally from the MCL and PCx when the rat was exploring the home cage is shown in Fig. 7. Averaged data showing power spectra at theta range (4-12 Hz range) calculated for the periods of exploratory locomotor activity from MCL and PCx in 70 and 120 days NC and OTT rats is given in Fig. 8. During the exploratory locomotion, there was a peak, evident at 4-12 Hz in the PCx (bilaterally) of the NC rats at all age groups. Clear peaks at 12-20 Hz were also present in PCx and MCL (right hemisphere) during spontaneous exploratory locomotion. A significant increase in the relative power at theta range was seen in PCx of 70 days OTT rats when compared to NC ($t_{1,16}=2.137; P<0.05$) (Fig. 8c). The difference in the theta peaks in MCL or PCx was not obvious between NC and OTT rats of 120 days group (Fig. 8 a-d). In addition, it was also observed that when the rat was exhibiting exploratory walking in the home cage, oscillatory activity at theta range was visible in both PCx, as well as in the MCL of olfactory bulb. However, the synchronized theta oscillation was more pronounced in the PCx than in the olfactory bulb of all the age groups. The synchronization was seen only when the rat was exploring the home cage, but did not occur when the animal was immobile.

Further to quantify the reinnervation of the Mitral cells with their target (Piriform cortex), coherence analysis was carried out. There was a significant decrease in theta coherence between the right
olfactory bulb and the right piriform cortex of 70 days OTT rats at 4-12 Hz ($t_{1,16} = 2.226; P < 0.04$) (Fig. 9). Reduction in the coherence was not evident in 120 days-OTT rats ($P > 0.05$).

**Discussion**

The present findings extend our understanding of regeneration in the central nervous system and further throw light on the lesion induced genesis of new Mitral cells and their connectivity with the target. The electrophysiological data revealed that neurons in Mitral cell layer of the olfactory bulb developed synchronized firing with piriform cortex after 120 days of olfactory tract transection. The present results also demonstrate the structural and functional restoration of the Mitral cells following early postnatal olfactory tract transection, although produced an initial degeneration of Mitral cells.

**Structural recovery of Mitral cells following bilateral olfactory tract transaction**—The olfactory bulb has a considerable degree of plasticity and has the capability to undergo successful regeneration following olfactory tract transection. In the present study, an initial exuberant growth of dendrites was observed within a distance of 40-80 µm by 70 days after the transection which could represent a possible mechanism involved in the reinnervation of Mitral cells with piriform cortex. By 120 days a gradual reduction in the dendritic intersections at distal segments of mitral cells was observed which was comparable to that of controls. This pattern of dendritic proliferation followed by pruning and ending with a single apical dendrite indicates an important phenomenon associated with the maturation and reinnervation of Mitral cells with its target. The presence of increased vascularity with enhanced dendritic sprouting in the bulb following OTT could also be associated with regeneration related changes. Finally, rapid Golgi stained sections along the line of transection strengthens an argument for a proper continuity in the central core of the olfactory tract indicating that rodent olfactory tract is capable of regenerating spontaneously following neonatal olfactory tract transection.

**Electrophysiological correlates of the home cage behaviour of the rat to demonstrate the functional integration of the Mitral cells with Piriform cortex following OTT**—Based on the available literature from the hippocampal and non-hippocampal studies, it is suggested that synchronization of the cortical neurons depends on the periodic activation of inhibitory interneurons and information processing. In the present study, decreased rhythmicity was observed at theta range within and between the olfactory bulb and piriform cortex after 70 days of OTT. Asynchronous relationship within and between the bulb and cortex, more predominantly with the right hemispheres during exploratory activity reflects the functional decoupling of the anatomical circuit at the site of the transection of olfactory tract.

![Fig. 8](image_url)  
**Fig. 8**—The effect of olfactory tract transection on theta frequency in the olfactory bulb and piriform cortex. (a) The average histogram showing the power spectral density (4-12Hz) from left olfactory bulb in the Mitral cell layer (MCL); (b) right olfactory bulb; (c) left piriform cortex and (d) right piriform cortex. Filled bars is from 70 days; hashed bars is from 120 days group. NC (n=8 for 70 day and n=7 for 120 day) and OTT (n=10). Value are mean±SE, *P < 0.05.

![Fig. 9](image_url)  
**Fig. 9**—Changes in LFP theta (4-12Hz) coherence from NC and OTT rats during exploratory behavior. (a) average LFP coherence between right MCL and right PCx at 70 days (b) 120 days (c) average coherence between left MCL and left PCx at 70 days and (d) 120 days group of rats. Data were compared between NC (filled box) and OTT (empty box) groups of rats. MCL, Mitral cell layer of olfactory bulb; PCx, piriform cortex; NC, normal control (n=8); OTT, olfactory tract.
The possible mechanisms that may be responsible for the desynchronized pattern of neuronal activity in both the bulb and the cortex following OTT could be due to the following reasons: one of the unique characteristic features of the olfactory cortex is that all the primary sensory inputs from the olfactory bulb are processed and filtered at the level of piriform cortex54 without the involvement of the thalamus as seen in other sensory systems55. The filtering of the incoming signals at the cortical neurons during exploratory behaviour can result in desynchronization with a decreased rhythmicity that may occur as a result of less entrainment of principal neurons either with their afferents or with local interneurons as indicated with higher theta power in the piriform cortex. This highlights the significance of filtering of afferent signals at piriform cortex56, resulting in the convergence of olfactory inputs, which is necessary for the discriminatory information processing.

Further a question which may be asked here is what may be the spontaneous behavioural consequences of the observed morphological and electrophysiological changes in olfactory bulb and piriform cortex. Odour-induced deficits have been reported in the behaviour after bulbectomy57, and lateral olfactory tract transection19, suggesting the lesion-induced memory deficits in tests based on olfactory cues. The present results also suggest that transection of olfactory nerve fibers leads to deafferentation of olfactory bulbs and a loss of olfactory mediated behaviour58. However, the present findings show the spontaneous behavioural recovery of the OTT rats in their home cage by 120 days. In general, OTT also led to certain behavioural enhancements, such as increased time spent in exploring and rearing behaviour in the home cage as compared to normal control rats.

Consideration of possible lesion-dependent changes in dendritic sprouting of afferent neurons and increased asynchronous firing in olfactory bulb and piriform cortex remains speculative. It is possible that OTT-induced dendritic re-modeling in the Mitral cells of the olfactory bulb after OTT could be one of the triggering factors for the regeneration of Mitral axons to re-innervate the piriform cortex. Lower coherence of LFP between MCL and PCx with recovery of the dendritic sprouting indicates the bulbocortical coherences probably driven by excessive excitatory inputs from Mitral axons into the piriform cortex.

Since the olfactory tract transection was bilateral and complete leading to total anosmia, an alternative mechanism exists to compensate for the functional deficit which is critically required for the survival of the animal. The present results have shown that the olfactory tract transected rats survived the axotomy and reached the experimental time points without any significant weight loss or food intake. The present study, thus, provides several potential evidences to support the compensatory replacement of the neural connections between olfactory bulb and piriform cortex following olfactory tract transection.

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