

# An Artificial Neural Network Model of the Milk-Ejection Reflex in the Sheep

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## Abstract

*Milk ejection reflex involves an ascending neuronal pathway from the nipples to the hypothalamus and a descending vascular limb. Anatomical data reveal that in sheep, ascending neural information passes through the medial cuneate nucleus (CUM), or the lumbar spinal segments (LSS) and the lateral cervical nucleus (LCN) before reaching the hypothalamic paraventricular nucleus (PVN). Physiological measurements suggest that although the nipples are periodically stimulated during suckling, the hypothalamic hormone oxytocin, is only released in the bloodstream once or twice.*

*In a first attempt to develop an artificial neural network of the above pathway, we model a typical relay station, namely the CUM, LSS or the LCN, as a sparsely connected network consisting of excitatory and inhibitory populations of leaky-integrate-and-fire (LIF) neurons. The network is characterized by its connectivity, the average number of connections for each neuron, and a parameter  $\gamma$  determining the ratio of excitation versus inhibition. Excitatory connections have modulated synaptic weights; strengthen upon neuronal firing and slowly decaying otherwise. The network responds to periodic stimulation with one or two bursts of highly synchronised neuronal activation and then enters a chaotic regime where naturally synchronization disappears. Such a relay station could provide the appropriate input for oxytocin release.*

## 1. Introduction

Suckling and milking are two stimuli inducing the neuroendocrine milk-ejection reflex. The latter involves an ascending neuronal pathway from the nipples to the hypothalamus and a descending vascular link that conveys pituitary hormones to the mammary gland.

Oxytocin, a hypothalamic hormone, is responsible for contraction of the myoepithelial cells surrounding the mammary alveoli. The pattern of oxytocin release exhibits species specific characteristics and can vary significantly [8]. The action of oxytocin after being released into the blood stream from the paraventricular (PVN) and supraoptic (SON) nuclei neurons following nipple stimulation, as well as the bursting activity of oxytocin neurons [4] have been investigated in depth. Extracellular recordings of oxytocin neurons in the PVN and the SON of the anaesthetized lactating rat have revealed that, for most of the suckling period, oxytocin neurons appear quite unresponsive to the suckling efforts of the pups. The neurons maintain a steady background discharge which is unaffected by the application or removal of the pups from the nipples. In contrast, just before each milk ejection, the neurons suddenly display a characteristic high frequency discharge of action potentials followed by a period of reduced firing or quiescence. Electrophysiological studies also exist to describe the milk-ejection reflex in the sheep [9] where oxytocin is

released only once or twice during each suckling session. The fact that oxytocin neurons are unaffected by the suckling efforts between successive milk ejections suggests the existence of a mechanism which somehow interrupts the input from the nipples before it reaches its hypothalamic destination [6]. There is a lot of interest in networking and/or modeling biological pathways emphasizing their interactive nature. An exhaustive analysis has been performed on the circuitry of the lower urinary tract [3]. From this respect, the milk-ejection reflex has not yet been investigated.

The present study is a preliminary attempt to design and develop a prototype neural network MESANN (Milk Ejection Simulation Artificial Neural Network) to be used in simulating the milk-ejection reflex in the sheep, based on anatomical data produced by horseradish peroxidase tracing of the pathway. In the lack of neurophysiological data concerning the ascending pathway relay stations, namely the CUM, LSS or the LCN, we model a typical relay nucleus consisting of a mixed population of excitatory and inhibitory leaky-integrate-and-fire (LIF) models receiving periodic stimulation. The output of the network is the activity of the excitatory neurons since they transmit information to the hypothalamic nuclei. We investigate possible mechanisms through which periodic stimulation can induce only a few highly synchronized activations providing the appropriate stimulus to the hypothalamic centers for oxytocin release.

## 2. Methods

### 2.1. Tracing the relay stations of the pathway

**2.1.1. Peroxidase injections in the nipples.** Experiments were performed on nonlactating, maternally experienced ewes (*Ovis aemon aries*,  $n=20$ ) of native colonies. Animals were housed on a normal light-dark cycle with food and water freely available. Under sodium pentobarbitone (a 10% solution; 30mg/kg i.v.) anesthesia, multiple injections of a 2% aqueous solution of wheat germ agglutinin (WGA) conjugated to horseradish peroxidase (HRP) were delivered intradermally in one or two udders. Injections covered the whole surface of the nipples in most of the animals. The number of the injections and the quantity of the injected peroxidase ranged by the size of the nipple

**2.1.2. Peroxidase injections into the lateral cervical nucleus.** Since previous studies [1, 2, 7] have shown that the LCN and the spino-cervico-thalamic tract are involved in the transmission of the sensory information from the nipples to the brain, peroxidase injections into this nucleus were employed. Multiple injections of a 30% aqueous solution of HRP or a 2% solution of WGA-HRP were delivered into the LCN ( $n=7$ ). In each animal the LCN was exposed to 3-5 rostrocaudal peroxidase injections following partial laminectomy of the first cervical vertebra. Approximately 0.5-1  $\mu$ l of peroxidase was injected each time with a 1- $\mu$ l Hamilton microsyringe, in a position located 2-3 mm from the dorsal medial sulcus of the spinal cord at depths of 2.0, 2.5 and 3.0 mm from the dorsal surface of the spinal cord. Subsequent injections were made 15 minutes after the previous so that the surrounding tissues could retract to their normal condition after the penetration of the syringe.

**2.1.3. Peroxidase injections into the paraventricular hypothalamic nucleus.** Peroxidase injections into the PVN were achieved after removing part of the frontal bone ( $n=7$ ). A 30% HRP aqueous solution was injected through the proreus gyrus (just before the cruciate gyrus) at a depth of 3.2 and 3.5 cm from the surface of cerebral cortex. Three to four mm lateral to the median longitudinal sulcus of the cerebrum with a 35° angle from the dorsal surface of the cerebral hemispheres, a 1- $\mu$ l Hamilton microsyringe delivered 0.3-0.5  $\mu$ l of peroxidase in each animal. Ten to fifteen min intervals were taken in between the injections at different levels.

**2.1.4. Perfusion and tissue processing.** In all cases, after survival periods of 72-96 hours, the animals were re-anaesthetized with sodium pentobarbitone and perfused through the ascending aorta with 7 liters of phosphate-buffer solution (PB, 0.1M, pH: 7.2) at room temperature (5-7 minutes) followed by 7 liters of 1.25% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer at room temperature (30 minutes).

Following the perfusion of the animal, the injection site and the medulla oblongata, the mesencephalon, the hypothalamus and the lumbar part of the spinal cord (in the case of LCN injections), or the medulla oblongata, the first two cervical segments and the lumbar part of the spinal cord (in the case of PVN injections) were dissected for examination. Following the peroxidase injections into the ewe nipples, the lumbar spinal ganglia, their corresponding spinal

segments and the medulla oblongata were also dissected. Tissue samples were stored in the same fixative for 4 hours at 4° C. Afterwards they were stored in 0.1M PB containing 5% sucrose at 4° C for 15-17 hours and finally into a 30% sucrose PB solution for 24 hours at the same temperature. Serial coronal or horizontal sections were cut using a freezing microtome (50µm). The coronal sections of the spinal segments were kept according to their rostrocaudal level (anterior, medial or posterior part of the spinal segment). The free-floating sections were subsequently reacted histochemically with tetramethyl-benzidine (TMB, Sigma) according to the procedure described by Mesulam (1982); [5]. They were then lightly counterstained with Neutral Red and examined with a Carl Zeiss photomicroscope using bright and darkfield illumination.

All experiments were carried out according to the guidelines of the animal experimentation ethical committee of the Aristotle University of Thessaloniki, conforming to European Union guidelines.

## 2.2. Single neuron models and network structure

The network is composed of  $N$  LIF neurons, from which  $N_E$  are excitatory and  $N_I$  inhibitory. Each neuron receives  $K$  randomly chosen connections out of which,  $K_E$  stem from excitatory neurons and  $K_I$  from inhibitory neurons within the cluster (local interneurons). Parameter  $\gamma$  defines the balance of excitation and inhibition in the station,  $\gamma=N_E/N$ . We consider sparsely connected networks with connectivity  $k=K/N < 1$ .

The depolarization  $V_i$  of neuron  $i$  ( $i = 1, \dots, N$ ) is described by the equation

$$\tau \frac{dV_i}{dt} = -V_i + RI_i(t) - I_{ext}(t) + \xi(t)$$

where  $I_i(t)$  are the synaptic currents arriving at the soma which can be further divided into excitatory  $I_{exc}(t)$  and inhibitory  $I_{inh}(t)$ .  $I_{ext}(t)$  is the external stimulus representing the nipple stimulation during suckling. The young is firmly attached to the nipples for prolonged periods and only give very brief sucks. External stimulation was therefore modeled as a train pulse with characteristic amplitude  $S_A$ , pulse duration  $S_D$  and period  $S_P$  with  $S_P \gg S_D$ . The term  $\xi(t)$  is a Gaussian noise input of zero mean and  $\sigma$  standard

deviation. When  $V_i(t)$  reaches the firing threshold  $\theta$ , an action potential is emitted by neuron  $i$ , and the depolarization is reset to the reset potential  $V_0$  after a refractory period  $\tau_{rp}$  during which the neuron is insensitive to further stimulation.

The synaptic currents are the sum of the contributions of spikes arriving at different synapses. Synaptic potentials are modeled as alpha functions in the basic LIF model:

$$RI_i(t) = \tau \sum_j w_{ij} \sum_k ((t - t_j^k - D) * e^{-\alpha(t - t_j^k - D)})$$

where the first sum on the right hand side is a sum on different synapses ( $j = 1, \dots, K$ ), with postsynaptic potential (PSP) amplitude  $w_{ij}$ , while the second sum represents a sum on different spikes arriving at synapse  $j$ , at time  $t = t_j^k + D$ , where  $t_j^k$  is the emission time of  $k^{\text{th}}$  spike at neuron  $j$ , and  $D$  is the transmission delay and  $\alpha$  determines the rate of the exponential. For simplicity all synaptic weights to a neuron have been considered equal  $w_{ij} = w_i$ . Inhibitory PSP amplitudes were taken constant, so  $w_i = W_I < 0$ . However, excitatory synapses exhibit a form of Hebbian plasticity. The change in the weight of a synapse is described by the differential equation:

$$\frac{dw_i}{dt} = -\frac{w_i}{\tau_w} + I_{\delta w} + W_E$$

where  $\tau_w$  is the time constant,  $W_E$  the base excitatory synaptic weight,  $W_E > 0$  and  $I_{\delta w}$  is a term estimating the contribution of action potentials to the  $w_i$  as a function of time:

$$I_{\delta w} = G_w (t - t_i^k) e^{-\beta(t - t_i^k)}$$

where  $G_w$  is a constant of increase in synaptic weight each time neuron  $i$  fires an action potential and  $\beta$  determines the rate of the exponential.

The parameter values used in our simulations were the following:  $N=100$ ,  $k=0.2$ ,  $\gamma=0.7$ ,  $\theta=1$ ,  $V_0=0$ ,  $\sigma=0$ ,  $\tau_{rp}=2\text{ms}$ ,  $\tau=5\text{ms}$ ,  $D=2\text{ms}$ ,  $a=5$ ,  $W_I=-5$ ,  $W_E=1$ ,  $\tau_w=100\text{ms}$ ,  $G_w=0.7$ ,  $\beta=1$ . Initial conditions for each unit were randomly assigned between  $V_0$  and  $\theta$ . In all simulations, we used the Euler integration scheme with a time step of  $\Delta t = 0.01$ . All simulation programs were coded in the C programming language and run on Pentium-based Windows xp® workstations.

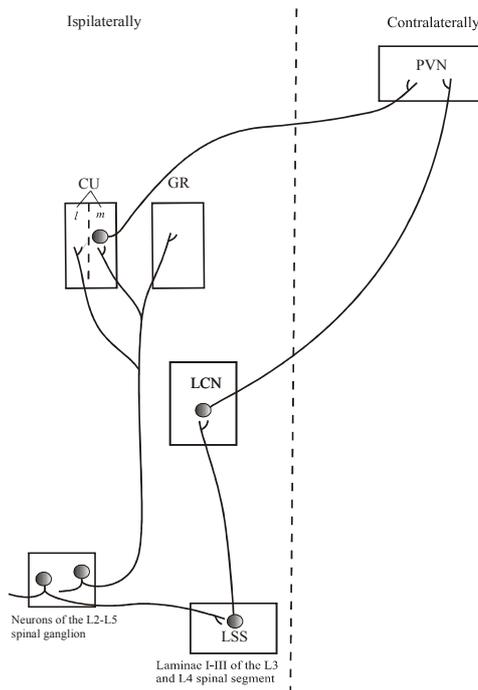
### 3. Results

#### 3.1. Unveiling the relay stations of the pathway

Injections of HRP in the nipples of the ewe resulted in labeled cells in the 2nd to 5th ipsilateral lumbar (L) dorsal root ganglia (DRG). Central projections of the DRG-labeled cells were found in the medial part of laminae I-III of the ipsilateral L3 and L4 spinal segments. Limited projections were also found in the medial cuneate nucleus in medulla oblongata.

Following the injections into the LCN, retrogradely labeled neurons were found in the spinal cord, the medulla oblongata, the mesencephalon and the hypothalamus. In the lumbar (L3 and L4) spinal segments, retrogradely labeled neurons were observed ipsilaterally to the injection site, in the dorsal horn (laminae I-IV), the intermediate gray matter and the ventral horn. A special group of neurons in the dorsal horn were encountered in the medial part of the laminae I-III.

Following peroxidase injections in the PVN retrogradely labeled neurons were observed in the contralateral LCN and the medial cuneate nucleus.



**Figure 1: Neuronal pathways from nipples to hypothalamus**

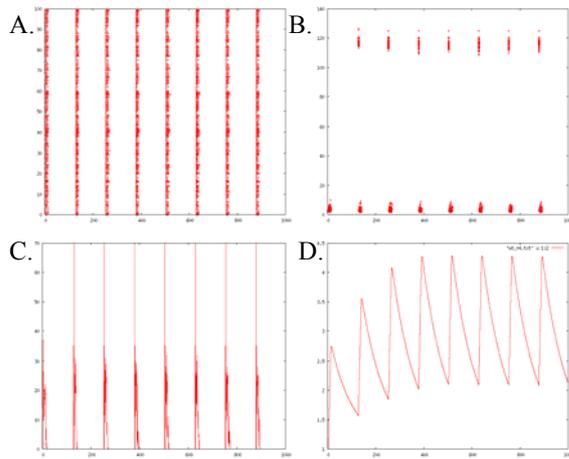
The above described anatomical results demonstrate that in the sheep, the transmission of the afferent input from the nipples to hypothalamus may be accomplished through at least two pathways that are crossed and polysynaptic (Fig. 1). Any relay station of the pathway may receive excitatory and/or inhibitory information from adjacent neurons. The possible existence of other parallel routes of transmission needs further investigation.

#### 3.2. Simulation

Initially amplitude modulations were considered in the  $I_{ext}(t)$ . It is known by the milking process that the pressure to be applied must not be too weak or too strong for a successful milking process. For the following,  $S_D$  and  $S_p$  will be constant and set to 12.5ms and 125ms respectively.

**3.2.1. Weak stimulus amplitudes.** For weak stimulus amplitudes the network responded with a moderate activation of the units. In figure 2A the response of the network is displayed for  $S_A=0.4$  by showing the firing times of all units in a diagram known as raster plot. In figure 2B the interspike time intervals (ISI) are displayed against time. The periodic nature of the response is again evident since two main groups of ISIs are distinguishable, one of large duration between the network burst and one of small duration during the bursts. A close examination will reveal that after an initial transitory phase all units fire perfectly periodically. In our model, synchrony of firing of the excitatory units is a major importance since they constitute the output of the network. We hypothesise that persistent synchronous firing of the excitatory units will boost the centers in the hypothalamus to release oxytocin. According to experimental data this release should occur only once or twice during a suckling period so we wish our network to provide strong synchronous firing of its excitatory units only once or twice. In figure 2C we provide a measure of synchrony where the activity of all excitatory neurons is measured in time bins of 1ms. As it can be seen, excitatory neurons fire synchronously only for a very small period in the order of 1ms, and then the value drops to significantly lower values. We consider that this level of activation is insufficient for proper hypothalamic activation; therefore weak stimulation fails to induce hormone release. In figure 2D the modulation of the synaptic weight of an exemplary excitatory unit of the network is shown (unit #: 0). The weight  $w_0$  starts from the  $W_E$  value ( $W_E=1$ )

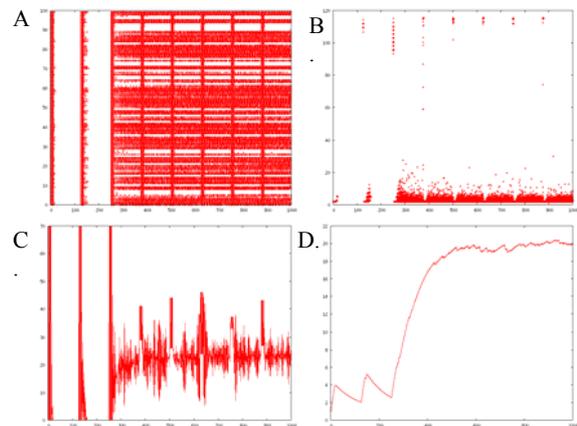
and during each stimulation cycle moves to higher values when the neuron fires. Thanks to the large value of its time constant  $\tau_w$ ,  $w_0$  slowly decays during quiescence. However, for this level of stimulation it reaches an oscillatory regime and varies between the values of 3 and 4.28. Excitation is not sufficient to preserve network activity during the absence of stimulation and the weak stimulus amplitude can not induce strong synchrony. Both factors are impeded by the action of local interneurons (30% of the network population) which also possess large absolute values of synaptic weight ( $W_I=-5$ ).



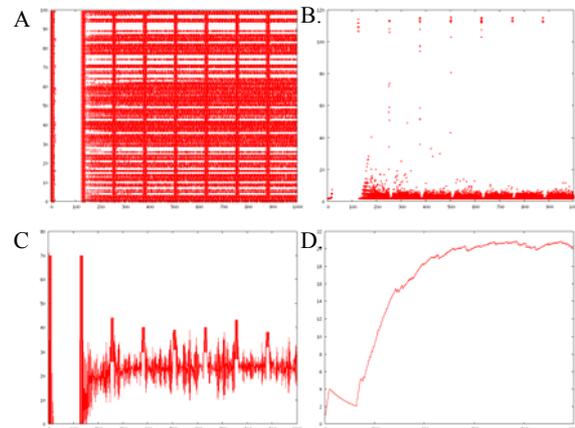
**Figure 2: Weak stimulus amplitudes**

**3.2.2. Moderate stimulus amplitudes.** As the stimulus amplitude increases the frequency of firing increases and excitatory synaptic weight takes on higher values. At the same time the frequency of inhibitory neurons increases and a point is reached where sustained network activity is observed but neurons display highly irregular firing, indicating a chaotic regime for the network. Figure 3 is completely analogous of figure 2 only now the stimulus amplitude is  $S_A=0.8$ . The raster plot in figure 3A shows three cycles of periodic and highly synchronized behavior before the network enters a regime where activity sustains after the stimulus offset but firing is highly irregular therefore not synchronized. Irregular firing is also evident in the ISI diagram in figure 3B by the large dispersion of ISI values (compare with figure 2B). Note that some units stay in the periodic regime. This inhomogeneity in the behavior is due to the fact that the number of connections a neuron receives is random. Naturally, neurons that happen to receive a small number

of connections are less affected by the global dynamics. Figure 3C quantifies synchrony for the network and verifies that synchrony was achieved for all neurons only during the first 3 cycles and also was more persistent than in the case of weak stimulation. Then, although the stimulus persists periodically the network never achieves this level of synchrony again. The synaptic weight of the exemplary unit (#: 0) instead of oscillating between two values is now able to escape the periodic regime and take on large values. In the chaotic regime the weight seems to stabilize around some value  $w_0 \approx 19$ .



**Figure 3: Moderate stimulus amplitudes**



**Figure 4: Strong stimulus amplitudes**

**3.2.3. Strong stimulus amplitudes.** For stronger stimuli the chaotic regime appears sooner. In figure 4 we

demonstrate the case for  $S_A=1$  where after a single cycle the network enters the chaotic regime and thereafter can not produce the desired synchronized activity to promote oxytocin release. Larger stimuli have similar results as the maximum frequency that can be achieved is the one imposed by the refractory period. The value of  $w_0$  settles in a value slightly larger than for  $S_A=0.8$  ( $\approx 20$ ).

#### 4. Conclusions

Unveiling the anatomical features of the pathway responsible for oxytocin release is one important step towards the understanding of its function. By modeling the ascending anatomical pathway, this study aims to provide a tool that can be useful in the investigation of the complex function of neural control of the milk let down and indirectly oxytocin release. Anatomical data reveal that the transmission of the afferent input from the nipples to hypothalamus may be accomplished through at least two pathways. The part of the first pathway up to the LCN is ipsilaterally located. The second part of the first pathway connects directly the LCN with the contralateral PVN, a route, which does not belong to any of the known afferent systems. The second pathway follows the route of the fibers of gracile and cuneate tract. The first part of this pathway terminates at the medial cuneate nucleus and the second part does not follow the typical route of the fibers of the medial lemniscus system.

A mixed excitatory/inhibitory network of LIF neurons was constructed; the MESANN. Upon periodic stimulation and for the appropriate stimulus amplitudes gives 2-3 synchronized bursts of activity before it enters a chaotic regime where sustained non-synchronized activity is observed. We expect such an output to be the one expected from the hypothalamic nuclei to promote hormone release. Weaker and stronger stimuli fail to produce the same behavior. Although we do not demonstrate it here, shorter and longer in duration stimuli will also fail to provide the necessary output again in agreement with experimental data. The mechanism is based on the progressive strengthening of excitatory synapses through synchronized bursts which lead to the chaotic not synchronized regime.

The possible application of this model in simplifying and making various experiments cost and time effective summarizes its usefulness. The presented model can be improved by deepening the knowledge of the neuroanatomical and physiological background of the

reflex. In particular, additional physiological data would permit us to restructure, train and test the model. The future use of this model will be helpful to assess the possibility of producing larger quantities of milk in a cost-effective way.

#### 5. References

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