The sensory and motor effects of etomidate anaesthesia.

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Abstract (251)

The effects of anaesthesia with etomidate on the cellular mechanisms of sensory processing and sensorimotor coordination have been investigated in the active electric sense of the mormyrid fish Gnathonemus petersii. Like many anaesthetics, etomidate is known to potentiate GABA_A receptors but little is known about the effects on sensory processing at the systems level. A better understanding is necessary for experimental studies of sensory processing, in particular regarding possible effects on the dynamic structure of excitatory and inhibitory receptive fields and to improve the knowledge of the mechanisms of anaesthesia in general.
Etomidate slowed the electromotor discharge rhythm, probably due to feedback inhibition at the premotor level, but did not alter the structure of the electromotor command. Sensory translation through primary afferents projecting to the cerebellum-like electrosensory lobe (ELL) was not changed. However, central interneurons and projection neurons were hyperpolarized under etomidate and their spiking activity was reduced. Although the spatial extent and the centre/surround organization of sensory receptive fields were not changed, initial excitatory responses were followed by prolonged inhibition. Corollary discharge input to ELL was maintained and the temporal sequence of excitatory and inhibitory components of this descending signal remained intact. Later inhibitory corollary discharge responses were prolonged by several hundred milliseconds. The result was that excitatory reafferent sensory input was conserved with enhanced precision of timing, while background activity was greatly reduced. Anti-Hebbian synaptic plasticity evoked by association of sensory and corollary discharge input was still present under anaesthesia, and differences compared with the non-anaesthetised condition are discussed.

INTRODUCTION

The possible bias introduced by the effect of anaesthetics is a general problem for systems level studies of brain processes in whole animal preparations (e.g. see Edeline 1999; Gottschalk 2003; Maclver and Kendig 1989). To date, the mechanism of loss of sensory perception during anaesthesia remains poorly known.

The electrosensory system of mormyrid electric fish is a particularly interesting model in which this can be studied because the pathways and physiology of corollary discharge signals which gate sensory integration are well known. The
corollary discharge is driven by an efference copy arising in the electromotor command nucleus and is a descending signal that actively filters on-going sensory input, in the manner of a central prediction based on recent sensory history correlated with electromotor strategy. It may also include multimodal sensory feedback from proprioceptive, visual or lateral line sensory systems. Here, we have examined the effects of the anaesthetic etomidate on the integration of sensory and corollary discharge signals, in order to better understand how etomidate affects both sensory processing in the initial stages of the central processing pathway.

The intrinsic network of the electrosensory lobe (ELL) is illustrated in Fig. 1. Corollary discharge input arrives via several pathways that terminate in different layers of this cerebellum-like network: from the juxtalobar nuclei, terminating in the granular layer, and from the nucleus preeminentialis either directly, terminating in the lower molecular layer, or indirectly via parallel fibres originating from Eminentia granularis posterior that terminate in the outer molecular layer (Bell and Szabo 1986; Maler 1973). The sensory responses of interneurons and projection neurons of the ELL are strongly influenced by the descending corollary discharge input, which creates a succession of inhibition and excitation timed precisely with the arrival of reafferent sensory input generated by the fish’s own electric discharge (Bell and Grant 1989).

Etomidate potentiates the action of GABA on GABA$_A$-receptors (Belelli et al. 2003; Rudolph and Antkowiak 2004; Yang and Uchida 1996b; Zhang et al. 2002) and for this reason can be expected to influence inhibitory synaptic activity in ELL, whose intrinsic network contains several different GABAergic microcircuits.
Most anaesthetics block the central electromotor command and because of this, until recently, the physiology of ELL neurons has been studied principally in curarized fish without anaesthesia. Curare blocks the activation of the electric organ, but not the central motor command, making it possible to study the effects of the corollary discharge in the absence of reafferent sensory input. This is an advantage for the study of central signals alone, and artificial sensory input can be generated by external point stimulation. However, sensory images generated in this way do not have the same spatial distribution or statistical structure as those generated by the fish’s own electric discharge.

Prior to using etomidate to explore natural electrosensory imaging, the aim of the present work has been to investigate how this anaesthetic affects sensory processing in this low level central network that shares many of the basic design principles of the cerebellum and the dorsal cochlear nucleus of mammals.

**Methods**

Nineteen mormyrid fish of the species *Gnathonemus petersii*, measuring from 10 to 17 cm in length, were used in electrophysiological experiments and three fish were used for non-invasive studies of changes in the electric organ discharge rhythm under anaesthesia. Fish were obtained from a registered dealer (Aquarélite, Aufargis, France) and housed in registered facilities conforming to French, European and international regulations concerning animal care (European Directive 86/609/EEC and the Treaty of Amsterdam Protocol on Animal Welfare (1997)). All experimental procedures were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources (N.I.H., USA).
**Surgery**

Induction of anaesthesia was obtained by immersion in an aerated solution of MS222 (3-aminobenzoic acid ethyl ester methanesulfonate salt, Sigma, St. Louis, USA; concentration 0.1 g/l). Following loss of postural equilibrium, artificial respiration was established with an aerated solution of MS 222 [0.06 g/l] administered through a tube inserted in the mouth, at a flow rate of 40 ml/min. Fish were held against a sponge with soft cotton threads, approximately 2.5 cm away from the back wall of the experimental tank, whose dimensions were 31x12x8 cm, length, width and depth respectively. A small piece of skull was removed above the electrosensory lobe and a Perspex cylinder was sealed around the skull-opening with denture relining cement (GC Reline, GC America, Alsip, IL, USA), closing wound edges which were first treated with lidocaine gel (AstraZeneca, Rueil Malmaison, France : Xylocaïne visqueuse 2%). The fish was then submerged approximately 1 cm below the water surface. Water conductivity was maintained constant throughout the experiment at ± 5 μS of a given value within the range 160 to190 μS. Following the end of surgery, MS222 was discontinued and fish were either curarized by intramuscular injection of 0.1 mg d-tubocurarine chloride (Sigma St. Louis, USA) or anaesthesia was continued with etomidate (Hypnomidate, Janssen-Cilag, Issy les Moulineaux, France) added to the respiratory water at a concentration of 1.6 μM, 2.8 μM or 4.1 μM. Etomidate at a concentration of 1.6 μM induced loss of postural equilibrium but some residual tail swimming movements remained and sometimes weak electromotor (but not skeletomotor) startle responses to unexpected sensory stimuli persisted. 2.8 μM etomidate resulted in the complete loss of motor reflex responses to external stimuli and 4.1 μM etomidate produced profound anaesthesia. At the end of the experiment fish were sacrificed with a lethal dose of MS222.
Data acquisition

The triple electromotoneuron volley known as the “command signal” was recorded with an Ag-AgCl wire placed around the electric organ in the tail. The first negative peak of the command signal is defined as “time zero” (Fig. 2A : $t_0$). This served as a temporal reference for data analysis and stimulation. The command signal was amplified (Cyberamp, Axon Instruments, Union City, USA) and fed to a trigger unit (Window Discriminator 120, WPI, Sarasota, USA) that generated a square pulse at the time of the first negative peak of the triple volley. This pulse was used to trigger data-acquisition, as well as the stimulator (Anapulse, WPI, Sarasota, USA) for the presentation of artificial electrosensory stimuli. In those cases where acquisition was not triggered by the command signal, data was recorded in continuous mode and cut into episodes off-line, starting at a chosen time before $t_0$ using Elphy data acquisition software (G. Sadoc, UNIC, CNRS, Gif sur Yvette, France).

Field potential recordings were made with low-resistance micropipettes (3M NaCl; 4-10 MΩ), recorded with an Axoclamp 2B amplifier (Axon Instruments, Union City, USA) (filter settings: DC - 3kHz). Before data was transferred to the acquisition software it was amplified and digitised (Cyberamp and DigiData 1323A interface, sampling rate: 10 to 250 kHz, Axon Instruments, Union City, USA). For current source density (CSD) analysis the recording electrode was advanced in steps of 25 μm. Field potentials were averaged over 20 to 30 episodes at each site. One dimensional CSD was calculated as described in (Gomez et al. 2005), using arc-tangent transformed data to enhance small events. Colour plots were constructed using MatLab (MathWorks, Natick, USA).
Intracellular recordings were obtained with microelectrodes filled with 2M potassium methyl sulphate containing 2% biocytin (Sigma-Aldrich, Lyon, France) (140-220 MΩ). To characterize individual cells, biocytin was injected intracellularly by passing depolarising and hyperpolarizing current : 0.1-0.6 nA for 2-10 min. Input resistance was measured at a delay of 80ms following time zero by passing square pulses of 10-20ms duration and 0.1nA amplitude. Acquisition of intracellular data was carried out as for field potential-recordings, with filters set at DC to 10kHz.

**Stimulation**

Electroensory stimuli were delivered to local areas of the skin through a pair of chlorided silver balls, 0.5 mm in diameter set 2.5 cm apart. The axis of the dipole was held perpendicular to the skin, with the negative electrode positioned closest to the skin. Stimulus duration was 0.1 ms and intensities were 2-20 μA.

For cells which increased their discharge rate when the receptive field centre was stimulated the normalized increase in discharge rate (NI) was defined as:

\[
NI = \frac{\text{discharge rate at centre of RF} - \text{spontaneous discharge rate}}{\text{discharge rate at centre of RF}}
\]

For cells that were inhibited by stimulation of the centre of the receptive field the normalized increase was defined as:

\[
NI = 1-\frac{\text{discharge rate at centre of RF} - \text{spontaneous discharge rate}}{\text{discharge rate at centre of RF}}
\]

**Histology**

At the end of experiments fish were deeply anaesthetised with MS222 (0.5g/L) and perfused with phosphate buffered saline (PBS) followed by 2%-paraformaldehyde and 2%-glutaraldehyde in 0.1 M PBS, pH 7.2. Following overnight fixation, brains were cut at 50-80 μm thickness in the transverse plane, on a
Leica VT 1000 vibratome (Wetzlar, Germany) and reacted with the ABC system (Vector Laboratories, Burlingame, CA). Labelled cells were reconstructed using either a drawing tube attached to a microscope (Leitz Orthoplan) or using the Neurolucida system (MicroBrightField, Williston, USA).

Statistics
Statistical comparisons are based on t-tests for paired data (StatView : SAS Institute, Cary NC, USA). If not noted otherwise, values concerning population data are given as means with standard deviations, with sample sizes indicated by a lower case n and the number of fish by an upper case N.

Results

Effects of etomidate on the EOD
To compare the timing of activity in different nuclei of the electromotor command chain and the electrosensory processing network, a zero time reference $t_0$ (see Fig. 2A) has been defined as the first negative peak of the electromotoneuron triple volley (the “command signal”) recorded at the skin above the electric organ (Bell and Grant 1992). The exact timing of the EOD relative to $t_0$ is of importance since the processing of reafferent sensory information in the ELL is gated by means of the EOCD arising from the electromotor command (Bell 1986, 1990, 1989; Bell and Grant 1992).

Figure 2A shows that etomidate did not alter the relative timing of $t_0$ and EOD. The latency of the positive peak of the EOD relative to time zero was averaged over 126 EODs before each change in the concentration of etomidate and compared to the latency observed in the absence of the anaesthetic. In the example shown in figure
2 the mean latencies of the EOD with 1.6 μM, 2.8μM and 4.1 μM of etomidate were 3.58 ± 0.03 ms, 3.55 ± 0.04 ms and 3.59 ± 0.04 ms respectively. After returning to respiration without etomidate, the EOD had a mean latency of 3.59 ± 0.05 ms. For the three fish investigated etomidate did not change the timing of motor events, as compared to the preparation without anaesthetic (Wilcoxon matched pairs test: p > 0.15, n = 126, N = 3).

The shape and the amplitude of the EOD were also unaffected by etomidate, as evaluated by comparing the integrals computed for the time window 1.76 ms on either side of the zero crossing separating the positive and negative peaks of the bi-phasic signal (Wilcoxon matched pairs test: p > 0.22, n = 126, N = 3).

However, etomidate did affect the rhythm of the EOD, which is normally instantaneously variable in un-anaesthetised fish, but became slow and regular under etomidate in a dose dependent manner (Fig. 2B). Averaging data from 3 fish, the mean inter-EOD interval increased from 90.8 ± 20.1 ms in the absence of etomidate to 1035.2 ± 61.8 ms in 1.6 μM etomidate, which was the working concentration used for intracellular recording. At concentrations greater than 4.1 μM the EOD sometimes split into 2 components, similar in form to the normal EOD but of different amplitudes; bursting of small, desynchronised EODs also occurred. After return to ordinary water the EOD returned to an irregular pattern within a few minutes.

In the unanaesthetised state, sensory stimuli could lead to a transient increase in the motor command rate known as a ‘novelty response’ (Post and von der Emde 1999). Such novelty responses were usually not observed at the anaesthetic concentration used in the physiological experiments (1.6 μM).
Field potentials and CSD analysis of corollary discharge responses

Field potentials and current sinks and sources evoked by the EOCD input are compared for an exemplary dataset in anaesthetised and un-anaesthetised fish in Figure 3. In the absence of etomidate, the earliest EOCD response recorded throughout all layers of the ELL, is a ramp-like positive wave (Fig. 3A: p1) (Bell 1990; Bell and Grant 1992). Following p1, n2 peaks as a sharp negative wave 5.5 ms after \( t_0 \) in the intermediate and granular layers (Fig. 3A). N2 is explained by a transient activation of cells in the granular layer of ELL by juxtalobar input (Bell and Grant 1992; von der Emde et al. 2000). A second brief positive wave (p2) is recorded from the plexiform to the molecular layer simultaneously with n2. It has been suggested that p2 represents the activity of the axon terminals of the granule cells, i.e. their terminals act as passive current sources (see below), and the presence of p2 up into the molecular layer can be explained by volume conduction (Bell and Grant 1992). In the plexiform layer, n2 fuses with a third negative going n3 (Fig. 3A) which has a latency of \( \sim 7 \) ms. Following n3 a broad positive wave (p3, arrow Fig. 3A and C) is recorded throughout the deep layers of the ELL. This wave peaks with a latency of about 15.7 to 17.3 ms in the ganglionic layer.

The CSD analysis (Fig. 3) reveals a first sink at the level of the intermediate and granular layers that reaches up to the plexiform layer, corresponding to field potential n2. The corresponding source extends from the plexiform to the molecular layer with a shortest latency of about 6 ms. A source corresponding to n3 can be seen in the plexiform layer with the corresponding sink reaching up to the ganglionic layer (* in Fig. 3A). A second and later sink/source constellation is characterized by the propagation of the sink (**) from the ganglionic layer into the molecular layer (\( \sim 200 \) to 380 \( \mu \)m).
In summary, it seems that early juxtalobar input excites cells in the granular layer and this activation invades the upper granular and plexiform layer leading to the activation of different cell types (~6 - 10 ms following $t_0$), resulting in the n2-n3 complex. Then action potentials propagate into the proximal molecular layer (Gomez et al. 2005).

Thirty minutes after the application of etomidate, the early components of the field potentials and CSD pattern in response to the EOCD input were in general quite similar to those observed in the absence of etomidate (compare Fig. 3A, B). However, the time course of the later components of the field potentials differed. The n3/p3 complex became sharper (Fig. 3C) and the duration of p3 (Fig. 3C arrow) was prolonged (measured as the width at 50% amplitude in the plexiform layer; Wilcoxon matched pairs test: $p = 0.018$, $z = -2.36$, $n = 7$). This resulted in a prolongation of the late sink in the molecular layer and the corresponding source in the granular layer.

In summary, both field potentials and CSD-profiles showed that etomidate did not disrupt the temporal sequence of synaptic events corresponding to the initial stages of sensory information transfer in the ELL. However, the later processes following the initial EOCD-input within the ELL are affected by anaesthesia, suggesting that etomidate does alter the balance of activity in the inhibitory circuits intrinsic to the ELL.

**Field potentials and CSD analysis of sensory inputs**

Primary afferent fibres are coupled electrotonically with postsynaptic granular layer neurons in ELL. Thus, in intracellular records from electroreceptor primary afferents, it is possible to see both the centrally conducting action potential of the afferent fibre itself, as well as synaptic potentials actually occurring in the
postsynaptic granule cell (Bell et al. 1989; Slesinger and Bell 1985), generated by central corollary discharge input or by sensory input from convergent primary afferent fibres. The mean corollary discharge EPSP latency (measured to the peak) in six primary afferents recorded in presence of etomidate was 4.9 ± 0.72ms. This is comparable to the corollary discharge EPSP latency of 5.4 ± 0.5ms reported in the presence of curare alone (Mohr et al. 2003a). Two of these primary afferents were recorded both before and during the application of etomidate (Fig. 4A) and no change was observed in the latency or amplitude of the corollary discharge EPSP, or the sensory EPSPs and spikes evoked in response to an artificial stimulus given at a longer delay, beyond the corollary discharge window. Field potentials and CSD analysis (Fig. 4 B, C) showed that the initial part of sensory responses in the upper granular layer to artificial stimulation at a delay of 80ms were not changed under etomidate. This was assessed by measuring the half width and peak latency of the field potential in the granular layer (Wilcoxon matched pairs test: p = 0.34, z = -0.94, n = 7). However, the later part of the negative sensory field potential was prolonged in two cases in the presence of etomidate. This increased the length of the sink centred at the granular/plexiform layer border and the corresponding source in the molecular layer. One reason for this might be that since etomidate prolonged the p3 component of the corollary discharge activity, the response to a late sensory stimulus occurring even at a delay of 80 ms may be superimposed on this.

*Effects of etomidate at the cellular level.*

Twenty three of the 24 cells analysed in the presence and absence of etomidate were stable enough to investigate alterations in their passive properties. On average, etomidate reduced spiking activity and produced slight membrane hyperpolarisation
control: -71.8 mV ± 7.5, > 10 minutes after etomidate application: -74.6 mV ± 8.7 mV; \( t \)-test for paired data: \( p = 0.003; \) \( df = 22, 23 \) cells; Fig. 5 B). Figure 5A illustrates the change in membrane potential distribution for different cell types which showed an overall hyperpolarizing shift. In the LF, LG and MG cells, secondary peaks appeared in the membrane potential distribution. These peaks were due to the development of a large IPSP in response to EOCD input, as described below.

Input resistance \( (R_{in}) \) decreased in the presence of etomidate in 16 of the 18 cells where this was investigated (Fig. 5B; before etomidate: 42.9 ± 22.4 MΩ; with etomidate: 29.4 ± 22.3 MΩ; \( t \)-test for paired data: \( p = 0.007, df = 19, 16 \) cells) and slightly increased in one MG cell and one LF cell. Following washout, the mean membrane potential and the input resistance were indistinguishable from the control levels (\( t \)-test for paired data: \( p > 0.35; \) \( df = 19, 8 \) cells).

These changes were accompanied by a reduced membrane potential variability (see Fig. 6 A-C) in all neurons (\( t \)-test for paired data: \( p < 0.014, df = 2499, 18 \) cells) except medium fusiform cells (4 of 4) and one of the eleven MG cells tested (\( t \)-test for paired data: \( p > 0.14, df = 2499, 5 \) cells). This was determined by comparing the time-locked inverse of the standard deviation of the membrane potential during the first 100 ms following time zero for both states in the individual cells. This method can reveal ‘clamping’ of the membrane potential due to underlying shunting inhibition even though no hyperpolarizing IPSP is visible (Monier et al. 2003)

Etomidate normally did not alter the general sequence of EOCD-evoked EPSPs and/or IPSPs, but the timing of the different excitatory and inhibitory components of the EOCD response could be shifted. In curarized, non-anaesthetised fish the earliest EOCD-response was an EPSP in 20 of the 23 cells (Fig. 6). However
in one MG-cell (Fig. 6B) and two LF cells (Fig. 6C, D) a weak IPSP preceded the EPSP. These IPSPs were enhanced and prolonged under etomidate in the LF cells while it was reduced in the MG-cell. In the LF-cells this delayed the EOCD-evoked EPSP by approximately 4 ms while in the MG-cell the EPSP peaked 3 ms earlier. These latency shifts and changes in the IPSPs were not due to membrane potential hyperpolarisation i.e. to changes in the EPSP driving force. This is shown for one LF-cell (Fig. 6D inset): When the cell was hyperpolarised by passing current through the recording microelectrode in the absence of etomidate (thick black trace), the early IPSP was not different from that seen at the resting membrane potential (thin black trace). Under etomidate (grey trace) both the initial and later IPSPs were potentiated and the latency of the interposed EPSP was shifted.

The most frequent change under etomidate was a reduction of the EPSP duration due to a potentiation of a late IPSP. This late IPSP was not always visible in absence of etomidate (Fig. 6A, B) but became prominent under anaesthesia. Its presence changed the \( V_m \) distribution to a two-peaked distribution (Fig. 5A). The time course of this late IPSP was further investigated using an analysis of membrane potential variation, calculated as the inverse of the standard deviation of time locked membrane potential for the period 30-100 ms after \( t_0 \). In two of 3 LF-cells and two of two LG-cells and nine of the eleven MG cells, the variability of the membrane potential was less than that in the control situation (\( t \)-test for paired data: \( p < 0.033 \), \( df = 1699 \), 13 cells). However, in all medium fusiform cells, one of the three LF cells, two MG-cells and the single TSD cell, there was no significant difference in membrane potential variability in the two situations (\( t \)-test for paired data: \( p > 0.281 \), \( df = 1699 \), seven cells). The TSD cell and medium fusiform cells were the only cell types where the late IPSP was never observed in presence of etomidate. We conclude
that in efferent and MG cells an EOCD-driven IPSP was enhanced under etomidate, that was less prominent in the absence of etomidate.

A consequence of the enhancement of the early and late IPSPs of the EOCD response was that in most cells the spiking probability was constrained to a short time following EOCD-input. The cross correlation (bin width of 2 ms) between the timing of action potential firing and the motor command signal was normalised by the number of motor-command cycles. The resulting distribution gives the probability that at a given time an action potential will occur following the motor command and the peak of this distribution was defined as the correlation coefficient. The probability that a given action potential is driven by the EOCD input was obtained by normalizing the cross-correlation distribution by the number of action potentials. The peak of this distribution gives the contribution index (Derjean et al. 2003; Usrey 2002). Three examples that demonstrate the major effects of anaesthesia on the spiking probability are shown in Figure 7. In eight of eleven cells the EOCD-coupled spikes occurred with a higher probability during anaesthesia, while the probability of later spikes was reduced (Fig 7A). This resulted in an increase of the correlation coefficient and the contribution index (Fig. 7A inset). In these cells the overall effect of etomidate was thus to increase the contrast between EOCD-evoked firing and background activity.

In two of seven MG-cells analyzed in this manner (Fig. 7B) spiking probability was reduced during the EOCD window. These cells differed from all other cells as spiking activity was markedly increased in the period following the long lasting IPSP, thus the contribution index decreased in these cells. No change in probability of EOCD-coupled spikes (Fig 7C) was encountered only in one LF-cell.
Since the tested population showed considerable variability, and because for each cell type the sample size remains small, no significant global changes (paired *t*-test: *p* > 0.16, df =10, eleven cells) could be identified in the contribution index, the correlation index or the latency of the EOCD coupled spikes (Fig. 7 D).

**Receptive Fields**

Electrosensory receptive fields of 11 cells (N = 7) were explored in the presence and absence of etomidate and characterised in terms of sub threshold properties and the spiking behaviour in response to artificial stimulation given at the reafferent sensory input timing. We compared the discharge rates, the normalized increase of the discharge rate in the centre of a given receptive field and the width of the receptive field, in anaesthetised and non-anesthetised conditions.

Figure 8 shows the raster plots of the spiking behaviour and also the sub threshold responses to an artificial local stimulus, for a medium fusiform cell (A), an LF cell (B) and an MG cell (C) in the absence (left) and presence (right) of etomidate. The stimulating electrode was moved from rostral to caudal along a straight line extending roughly from the tip of the Schnauzenorgan to the operculum of the fish (see fish in Fig. 8A) and passing in front of the centre of the receptive field of the recorded cell. In the control situation, in all cells except LG cells, stimulation in the centre of the receptive field caused an increase in action potential firing and decreased spike latency, compared to the response to the EOCD input alone. In the line-plots shown in figure 8 the average discharge rate measured at the different positions along the body surface, relative to the maximal rate measured in the centre of the receptive field is shown for the three cells.

Medium fusiform cells only show a sub-threshold EPSP in response to EOCD input but fired a single spike in response to reafferent sensory stimulation. There was
thus a sudden jump in the discharge rate (Fig. 8 A,B) as the stimulus passed through the centre of the receptive field. In contrast, the receptive fields of both efferent cells (Fig. 8 C,D) and MG-cells (Fig. 8 E,F) were characterised by rather smooth changes in discharge rates and spike latencies. Action potentials elicited from skin areas bordering the receptive field centre occurred with longer latencies than those elicited from the centre. Stimulation in the region surrounding the receptive field centre could reduce the number of action potentials and increase their latency, indicating the presence of an inhibitory surround. Such complex receptive fields were found in two of three LF cells and in two of four MG-cells.

Anaesthesia did not change the receptive fields of the three medium fusiform cells that were studied (Fig. 8A): the discharge rate, latency and width of the receptive field were unchanged. The receptive fields of all three LF cells, the LG-cell and two of the four MG-cells that were tested were also very little changed under etomidate although the LF cell illustrated in figure 8C showed a slight caudal-shifting of the receptive field centre under anaesthesia. The most notable effect of etomidate on receptive field organisation, observed only in MG cells (2 out of 4), was a marked increase in contrast between the centre and the inhibitory surround. The example shown for this group (Fig. 8 E,F) cell showed an opposite shift of the centre of the receptive field than the LF-cell illustrated in Figure 8D and E.

To what extent the shifts in the centre of the receptive fields can be attributed to the anaesthesia, or to plasticity of sensory responses caused by repetitive association of the sensory stimulus with the EOCD input, is difficult to evaluate. Similar shifts in the position of the receptive field centre were found in four cells for which we repeated the receptive field measurements in quick succession in the absence of anaesthesia. This indicates that plasticity rather than anaesthesia may be the probable
explanation and indeed, previous in vivo experiments (Bell et al. 1997b) have shown that repetitive association of a given sensory input with the corollary discharge produced plastic changes in the response to the latter after less than 10 associations.

For all ten cells investigated, Figure 9A summarizes the effect of anaesthesia on the width of the receptive fields. The receptive field width was not altered by etomidate (Wilcoxon-test, $z = -0.76, p = 0.43$, ten cells). However the stimulus driven response at the centre of the receptive field was increased by anaesthesia (Wilcoxon-test, $z = -2.36, p = 0.018$, ten cells), although this apparent effect is mainly due to the overall reduction in background firing activity.

In two LF cells and one LG cell we were able to repeat the receptive field measurements at a higher dose of etomidate [4.1 μM] corresponding to very deep anaesthesia. Under these conditions the width of the receptive field was considerably reduced and the normalized increase in discharge rate at the centre of the receptive field was even higher (data not shown).

**Plasticity**

Repetitive association of the same sensory input with descending EOCD input leads to plastic modulation of the effect of the EOCD (Bell et al. 1997b). It has been postulated that the EOCD input to ELL generates an internal “expectation” of sensory future in the form of a negative image of recent re-afferent responses, and that this acts as an adaptive filter of current re-afferent sensory responses (Bell et al. 1997a; Bell 1981; Bell et al. 1992; Roberts and Bell 2000). The effect of this filter would be to reduce the effects of sensory input that can be predicted from recent association. We investigated plasticity under etomidate by pairing the EOCD response with an artificial sensory stimulus evoked at the normal reafferent signal delay for 11 cells, of
which five (one LF, one TSD, two MG and one LG cell) were also investigated in the absence of the anaesthetic.

Examples of the plasticity obtained in presence of etomidate are shown for 2 MG cells and an LF cell in Fig. 10. MG cells fire two sorts of action potentials: small spikelets which are probably axon spikes that do not invade the soma, and large, broad action potentials that back-propagate into the apical dendritic tree. The latter have been implicated in the expression of plastic changes in responses to molecular layer EOCD input \textit{in vitro}. An example of pairing a sensory stimulus with the EOCD input in an MG-cell is shown in Fig. 10A, where the sensory stimulus inhibited the first spikelet evoked by the EOCD-input and at the same time increased the latency of a second spikelet. At the end of pairing, the inhibition of the first spikelet was becoming less complete and latency of the second spikelet had decreased. When the stimulus was turned off, the probability of firing an early spike was greatly increased compared to the pre-pairing period. The second spikelet was also evoked more frequently, although with a longer latency and more variable timing than during the pairing itself. Broad action potentials (grey dots) also appeared with a greater probability and these changes persisted for more than five minutes. Comparison of the intracellular responses shows an increase in the slope and amplitude of the EOCD EPSP following the pairing. A second MG-cell is shown in Figure 10B. This cell was inhibited by EOCD input and fired only a single broad spike in response to an artificial reafferent sensory stimulus. During pairing, the latency of this broad action potential increased and following pairing, it can be seen that the two components of the EOCD-evoked IPSPs were enhanced.

Figure 10C illustrates associative plasticity in an LF efferent neuron which was mainly inhibited by EOCD input. During pairing the sensory stimulus the cell
fired a burst of spikes whose latency gradually increased as pairing progressed. The latencies of the 2nd and later spikes of the sensory response increased more than that of the 1st spike and as pairing progressed, the later action potentials began to drop out. Following pairing, it can be seen that the slope and amplitude of both components of the EOCD IPSP had increased.

These results show that the mechanism of associative plasticity is not abolished by anaesthesia, although the increase in inter-EOD intervals that is induced under etomidate will necessarily affect the frequency of association.

**Discussion**

*Motor-system*

Etomidate did not alter either the timing of the EOD relative to the motor command, or the form and amplitude of the EOD. This means that the physical properties of the electric image projected on to the sensory surface are not changed and this is important to future studies of natural electrosensory imaging under etomidate anaesthesia. However, etomidate did have a major effect on the electric discharge rhythm, which was markedly slowed. The likely sites for this action are the medullary command generator, consisting of the relay and command nuclei, or the precommand nucleus (PCN) in the midbrain, which is the primary source of descending input to the command nucleus.

Under metomidate, a molecule similar to etomidate, the after-hyperpolarisation of command neuron action potentials was markedly prolonged as the inter-EOD period lengthened (Clausse and Grant 1986). However no recurrent synaptic inhibition in command neurons were found and immunohistochemical studies targeting anti-GABA or anti-GAD activity in the command and relay nuclei
have always given negative results. It remains to be investigated whether the effect of etomidate is due to potentiation of an as yet unidentified inhibition afferent to the command neurons, or whether the effect is indirect, acting by reducing descending excitatory input.

Much of the afferent input to the command nucleus comes from the precommand nucleus whose neurons are densely covered by GAD-positive terminals (Grant et al. 1999) and which receive a powerful corollary-discharge driven feedback inhibition (von der Emde et al. 2000). In another mormyrid fish, *Brienomyrus brachyistius*, (Carlson and Hopkins 2004) have shown that iontophoresis of GABA into the precommand nucleus lengthens the inter-EOD interval and that injection of glutamate. It is therefore likely that etomidate slows the electromotor rhythm by potentiation of corollary-discharge driven GABAergic inhibition of the precommand nucleus, with a consequent reduction of descending excitatory input to the medullary command nucleus. A direct action of etomidate on premotor circuits has also been described at several sites in the mammalian brain (Zeller et al. 2005; Zhang et al. 2001).

Sensory system

Primary afferent input was not changed by etomidate, suggesting that peripheral electroreceptor organs and sensory transduction were not affected. However, once within the electrochemical lobe, sensory integration can be subject to lateral inhibition and also depends strongly on motor context, i.e. on coincidence, or not, with corollary discharge feedback signals.

For sensory responses to stimuli falling outside the corollary discharge window, CSD analysis generally showed no changes in the first stage of central
integration of electrosensory input in the granular cell layer. At this level, primary afferents contact granule cells with electrotonic synapses and stimulus intensity is coded by both spike latency and the number of spikes in the afferent volley (Bell 1990, 1989). It might be expected that etomidate would potentiate lateral inhibition amongst granule cell populations, generated through connections with GABAergic large myelinated interneurons (Han et al. 2000a; Meek et al. 2001) and could thus alter the balance of the centre-surround structure of receptive fields. However, although potentiated lateral inhibition arriving one (chemical) synaptic delay later might curtail the integration of the later spikes of the primary afferent volley, it probably would not alter the coding of local stimulus intensity represented by the timing of the first spike of the primary afferent sensory response (Gomez et al. 2004).

A more complex situation is present in the case of reafferent sensory input which coincides with the corollary discharge gating window. Preliminary records (Engelmann and Bacelo, unpublished results) indicate that GABAergic large myelinated interneurons (LMI), present in the granular and intermediate cell layers, fire a single spike at the time of the electric organ discharge. The axon and dendrites of LMI cells synapse with granule cell bodies over a wide area (Han et al. 2000a; Meek et al. 2001), and it is therefore possible that this sets up a basal level of GABAergic inhibition over the granule cell population, coincident with the arrival of reafferent electrosensory input. Excitatory sensory responses are superimposed on this. In this case, by potentiating LMI inhibition of granule cells, etomidate could alter the threshold for granule cell integration of sensory input. More experiments and modelling studies will be necessary to understand the balance of excitation and inhibition in the granule cell layer and the full complexity of inhibitory gain control of sensory integration at this level.
Corollary discharge input

Field potentials and CSD analysis suggested that there was little change to the early components of the EOCD input to ELL under etomidate. We therefore conclude that the principal central inputs to the ELL, coming from the preeminential and juxtalobar nuclei and the caudal lobe parallel fibre pathway, are largely unaffected. This is of major importance regarding the use of etomidate anaesthesia for studies of sensory processing in the ELL, where integration of sensory input depends on the precise timing and complex temporal structure of the EOCD and primary afferent signals.

The visible effects of etomidate appeared in the later components of the corollary discharge responses, for example affecting the n3 and p3 complex of the EOCD field potentials which were significantly prolonged by etomidate. This prolongation of field potentials coincided closely with the timing of the late IPSP seen in intracellular recordings from ganglionic layer neurons. These components of the field potentials most likely represent intrinsic processing of the EOCD-input by cells within ELL, rather than late-arriving EOCD input (Mohr et al. 2003b).

Under etomidate, the importance of inhibitory connexions within ELL became very obvious in almost all neurons. Synaptically mediated IPSPs increased in amplitude and duration and in some cases, synaptic inhibition became apparent where none was visible before. An increase in synaptic inhibition under etomidate has now been described for specific cell types in a number of preparations including hippocampus, cortex and cerebellum in culture (Jurd et al. 2003; Wafford et al. 2004; Yang and Uchida 1996b), targeting postsynaptic phasic GABA_A-receptors. An increase in the probability of receptor channel opening and prolonging opening time is
probably mediated at β2 and β3 sub-type receptor sites (Angel and Arnott 1999; Proctor et al. 1986; Yang and Uchida 1996a; Zhang et al. 2002). Intracellular records from the present experiments also showed that under etomidate almost all cells in ELL became hyperpolarized, intrinsic spiking activity was reduced and input resistance decreased. These effects are consistent with an enhancement of tonic inhibitory conductance at extra-synaptic GABA_A receptors (Bieda and MacIver 2004; Farrant and Nusser 2005), possibly due to an increase in GABA overflow from the very large number of inhibitory synapses present in the molecular and cell layers of ELL. Tonic inhibitory conductances mediated by the α5 GABA_A receptor sub-unit in hippocampal pyramidal neurons are highly sensitive to etomidate and their enhancement has been associated with the amnesic effect of this drug (Caraiscos et al. 2004). However, in different cell types, tonic conductances can also be carried by other GABA_A receptor sub-units, for instance by the δ receptor sub-types in cerebellar granule cells (Farrant and Nusser 2005; Mody and Pearce 2004). It is not yet known which receptor sub-types are present in the different types of neurones in ELL but since most neurons are affected in this way by etomidate it is likely that tonic GABA_A conductances are widely expressed.

The most prominent change observed in MG cells and efferent neurons was the amplification of the late IPSP evoked by EOCD input. In cells of this type where no IPSP was initially visible, membrane potential showed reduced variability during this period and thus when an IPSP appeared under etomidate it is likely that this was indeed an amplification and not an IPSP generated “de novo”. The most likely source of this IPSP is from MG cells which represent 70-80% of the cell population in the ganglionic and molecular layers of ELL and are driven by EOCD input. MG-cells respond with bursts of small action potentials to the EOCD and these bursts begin 5 to
10 ms before the onset of the late IPSP seen in projection neurons and MG cells themselves. Their axons have a GABAergic terminal arborisation densely covering the soma of neighbouring output neurons (LG and LF neurons) within a span of about 300 microns either side of their cell body and probably also reciprocally contacting neighbouring MG cells (Meek et al. 1996). Mohr et al. (2003b) found that stimulation of the preeminential nucleus, a source of EOCD-driven feedback to ELL, evokes early excitatory responses in both efferent cells and MG-cells and that these are followed by late IPSPs of long duration. These authors favoured the explanation that the EOCD-driven IPSP seen in ELL was generated di-synaptically via MG-cell microcircuits activated by excitatory input from the preeminential nucleus.

Mohr et al. (2003b) also noted that the only cells in ELL that did not show an IPSP in response to nucleus preeminentialis stimulation were the TSD and medium fusiform cells. In our records these were also the only cell types that never showed a late, long lasting IPSP under etomidate. However these neurons were often hyperpolarized under etomidate, suggesting the presence of tonic GABA_A receptors.

Despite increased inhibition under etomidate, the major components of the sub-threshold EOCD synaptic responses were still present in the anaesthetized fish. The principal effect of increased inhibition was to reduce the time window in which ELL neurons were able to fire an action potential in response to excitatory EOCD and reafferent sensory input. This gave the impression of reduced jitter and increased precision in spike timing. Together with the overall reduction in intrinsic activity, the result was an apparent increase in the contrast of excitatory responses to EOCD or reafferent sensory input, which stood out against the following increased inhibition and tonically reduced background activity.
Similar results have been reported for a variety of anaesthetics and in different preparations (Edeline 1999; Kisley and Gerstein 1999; Massaux et al. 2004). In many studies a reduction in cortical activity has been described at anaesthetic doses lower than those affecting sensory evoked responses in thalamus or other low-level sensory nuclei (Antkowiak and Heck 1997; Antkowiak and Helfrich-Forster 1998; Antkowiak and Hentschke 2000; Antkowiak et al. 1997; Antkowiak and Kirschfeld 2000; Armstrong-James and George 1988; Bieda and MacIver 2004; Dougherty et al. 1997; Erchova et al. 2002; Gaese and Ostwald 2001), including experiments with etomidate (Angel and Arnott 1999; Pawelzik et al. 2003; Proctor et al. 1986).

**Sensory receptive fields**

The spatial structure of sensory receptive fields within the ELL is probably largely a property of the organisation of primary afferent projections and intrinsic connections within ELL and is not primarily dependent on descending input to the ELL. The phenomenon of lateral inhibition in the mormyrid ELL under conditions of natural stimulation has not yet been studied in sufficient detail, making it difficult to give a functional interpretation of the possible effects of potentiation by etomidate. However, the importance of lateral inhibition in defining receptive fields, in controlling information transfer and in enabling stimulus discrimination has been demonstrated in the rather similarly organized ELL of wave-emitting gymnotid electric fish *Apteronotus* (Bastian et al. 2002; Chacron et al. 2003).

The reported actions of anaesthetics on the size of receptive fields vary depending on the animal species, the chemical and pharmacological nature of the anaesthetics, the brain area and the sensory system investigated, and remains a topic
of much discussion that has been well reviewed by Dougherty et al. (Dougherty et al. 1997).

In the present study of ELL, neither receptive field width nor the centre-surround organisation (if present) were altered under a moderate depth of anaesthesia with etomidate. However, because intrinsic spontaneous firing was reduced, etomidate frequently increased the signal-to-noise ratio measured in the centre of the receptive field. At rather higher concentrations of anaesthetic the receptive field width increased, together with a further increase in the signal-to-noise ratio. This dose-dependency is in accordance with results obtained under etomidate anaesthesia in the rat (Angel and Arnott 1999).

**Synaptic Plasticity**

Our data demonstrate that under etomidate anaesthesia, plasticity was still present and this plasticity led to changes in synaptic efficacy in all MG-cells tested, as well as in LF cells. Thus, even though an *in vitro* study (van den Burg et al. unpublished results) showed that the probability of backpropagation of dendritic action potentials, that are necessary for the generation of parallel fibre synaptic plasticity in MG-cells (Han et al. 2000b), can be greatly reduced by etomidate, such plasticity can be induced *in vivo*. Since etomidate slows the EOD cycle, the repetition rate of EOCD-sensory associations is markedly reduced and it is likely that this change in frequency compared to the unanaesthetized animal, and the consequent increased interval between successive associations, is responsible for the differences in plasticity observed under anaesthesia. However, the basic mechanism of associative plasticity clearly remains intact.
In conclusion, it appears that while etomidate anaesthesia markedly reduces motor activity, it has only a relatively small effect on the processing of reafferent sensory input in the early central stages. This anaesthetic does not alter the basic structure of sensory responses or of corollary discharge-driven active filtering of sensory integration which provides the mechanism for distinguishing reafferent and efferent sensory input in the mormyromast region of ELL. However, sensory input occurring at times other than reafferent input generated by the fish’s own electric discharge will arrive during the potentiated inhibition that follows the initial facilitation set up by the corollary discharge-mediated active filter and therefore response threshold is likely to increase. The mechanisms for synaptic plasticity implicated in the formation and update of central sensory expectation remain intact.

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**Figure Legends**

Fig. 1 : **A** Section through the ELL indicating parallel fibre input to the molecular layer (white) and the mormyromast electrosensory afferent input to the granular cell layer (black). **B** Enlarged schematic view of the ELL showing several (but not all) different cell types based on morphological reconstruction following labelling with biocytin. Corollary discharge input arrives via: i) parallel fibre input to the outer molecular layer, ii) nucleus preeminentialis axons ending in the inner molecular layer and iii) juxtalobar nucleus input to the granular cell layer. Mormyromast primary afferents convey sensory information to the granular cell layer. Abbreviations: EGp: Eminimentia granularis of cerebellar caudal lobe, gang: ganglionic layer, gran: granular cell layer, plex: plexiform layer, inter: intermediate layer, mol: molecular layer. Interneurons – MG: Medium Ganglionic layer cells (GABAergic), MF: medium fusiform cell (GABAergic), TSD: thick smooth dendrite cell (non-GABAergic); G: Granular cells (probably GABAergic). Projection neurons (glutamatergic) – LG: Large ganglionic layer neuron, LF: Large fusiform neuron.

Fig. 2 **A-B**: Influence of etomidate on the shape and timing of the EOD relative to time zero (t0) (**A**) and the EOD-interval (**B**). **A** Upper traces show the biphasic electric organ discharge (EOD) and lower traces show the same signal amplified to reveal the
preceding triple electromotoneuron volley (the command signal). For each concentration of etomidate an average (n = 126) of the command signal and of the EOD is shown. The dashed vertical line indicates t₀ and the delay to the positive peak of the EOD is given for each condition. B The inter-EOD interval of a fish exposed to etomidate varied as a function of anaesthetic concentration (shown in μmol). When active motion stopped, respiration was assisted by intra-buccal perfusion.

Fig. 3 A-C: Field potentials and CSD plot derived from the field potentials. The arrows point to particular waves of the field potential referred to in the text. A Field potential and CSD plot in a curarized fish in response to the corollary discharge input alone, in the absence of sensory input. Time zero is indicated by the solid black triangle below the plot. B field potential and CSD obtained from the same fish after the application of etomidate. C Field potentials recorded in the plexiform layer of a second fish before (red) and during (black) the presence of etomidate.

Fig. 4 A-B: Effect of etomidate on primary afferents. A Spiking response (five traces superimposed) of a primary afferent, before (left) and after the application of etomidate. This cell was tested with a local artificial stimulus applied 40 ms following time zero. In this and the following figures the filled triangle represents time zero and open triangles represent the timing of the artificial local stimulus. B Field potentials, shown in black, overlaid on Current Source Density (CSD) colour plot of the response to a local sensory stimulus delivered with a latency of 80 ms following time zero. The CSD sink/source calibration is shown to the right of the figure. Responses were recorded in steps of 50 μm from the molecular layer down to the deep fibre layer of the ELL. For orientation, a medium ganglionic cell is shown relative to the different
layers of the ELL (mol = molecular layer, gang = ganglionic layer, plex = plexiform layer and gran = granular layer). C Response to the same sensory stimulus in the presence of etomidate.

Fig. 5 A-B: Summary of the effect of etomidate on the passive membrane properties (A, B). A Examples of histograms of the distribution of the membrane potentials of a medium fusiform cell, an LF cell and an MG cell. Histograms are based on 100 seconds of continuously recorded data in the absence (light grey) and presence (black) of etomidate. B Box-plot (mean, 10, 25, 75 and 90 percentiles) of the input resistance and membrane potentials recorded in presence (black) and absence (light grey) of etomidate.

Fig. 6 A-G: EOCD-responses and the effect of etomidate on the noise level fluctuations of the membrane potential ($V_m$). The original traces in A-C show the averaged EOCD responses of 2 MG interneurons (A and B) and an LF efferent neuron (C) under curare alone (black traces) and in the presence of etomidate and curare (white traces), with their corresponding standard deviation shown as a grey or black envelope respectively. The inverse of the standard deviation ($1/\sigma$) is shown for all three examples below the original traces (black for etomidate, grey for control condition). D-G show averaged traces of the responses of a second LF cell (D) and a LG cell (E), and two different interneurons: a TSD cell (F) and a medium fusiform cell (G), to EOCD input under curare alone (black traces) and under curare with etomidate (grey traces). The inset in D shows the early parts of the EOCD-responses at an expanded time scale, superimposed for different conditions: in presence of etomidate (thick grey trace) and before etomidate (thin black trace). The thick black
trace was obtained with artificial hyperpolarisation to the same membrane potential as measured in presence of etomidate. Calibrations at the left bottom of each set of records represent 20 ms; the filled black triangle indicates time zero as defined in the text. Reconstructions of the recorded cells, following labelling with biocytin, are shown to the left of each set of records.

Fig. 7 A-D: A-C: Post-t₀ spike-probability for two LF cells (A, C) and one MG-cell (B). Each probability is based on 100 sec ongoing activity in presence of etomidate (black) and without anaesthesia (grey). The insets show the cross correlation index (binwidth 1ms) between the motor command and the neuronal responses, normalized by the number of intervals, i.e., the contribution distribution. The contribution distribution on top is obtained in absence of etomidate and the distribution below in presence of etomidate. D Summary of the effects of etomidate (black bars) on the spiking behaviour of ELL neurons evoked by corollary discharge input, expressed as the correlation coefficient (CC), the contribution index (CI) and the latency of the peak in the cross correlation, compared to the same measures in the absence of etomidate (light grey bars).

Fig. 8 A-D: Receptive fields of a medium fusiform (A,B), an LF (C,D) and an MG-cell (D,F) in the absence (left) and presence (right) of etomidate. A,C and E: Colour-coded time course of the sub threshold membrane potential with superimposed raster plots of action potentials showing the changing response to an artificial dipole stimulus given at the EOD delay, moved in 2 mm steps, from rostral to caudal passing through the centre of the receptive field (for position of stimulating electrodes, see fish inset). The EOCD-response alone, in the absence of any sensory stimulus, is
shown at the bottom and at the top of each panel (separated by white dotted lines). Depending on the cell, 16 to 25 stimulus repetitions are shown at each location, resulting in a cumulative raster diagram. The broad action potentials of the MG-cell in E are shown by white dots, while the spikelets are indicated by black dots. **B, D and F:** Normalized discharge rate of the cells shown to the left. Blue curves indicate the non-anaesthetised state and yellow curves show the anaesthetised condition. The area under the curve corresponding to the 50%-criterion used to determine the width of the RF is shown by the filled part of each receptive field for the MG-cell and the LF-cell, while the width was measured over the total range of positions where stimulation evoked spikes in case of the medium fusiform cell.

**Fig. 9 A-B:** Summary graphs of the effect of etomidate on the width of the receptive fields (A), and (B) the normalized increase of the neuronal response. Note that values closer to 1 indicate an improved response.

**Fig. 10 A-D:** Plasticity of synaptic responses in presence of etomidate. **A-C** Pairing of sensory input at the EOD delay, with EOCD responses in three MG-cells. The raster-plots show the activity before, during (time indicated by gay bar) and after pairing. Grey dots: broad spikes; Black dots: small spikelets. The averaged time course of the membrane potential is shown for the 10 EOCDs prior stimulation (black) and just after the stimulus was turned off (grey) beneath each raster diagram. **D** Pairing in an LF-cell, the graphs are arranged as in A-C. Scale Time zero is given by black triangles; time of stimulus is given by open triangles.
Figures

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