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# Oxytocin enhances the inhibitory effects of diazepam in the rat central medial amygdala

D. Viviani [a], T. Terrettaz [a], F. Magara [a], R. Stoop [a,b,*]

[a] Centre for Psychiatric Neurosciences, Department of Psychiatry, University Hospital Center Lausanne (CHUV), Prilly-Lausanne, Switzerland
[b] Institute of Physiology, University of Lausanne, Switzerland

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ABSTRACT

Oxytocin is a neuropeptide that can reduce neophobia and improve social affiliation. In vitro, oxytocin induces a massive release of GABA from neurons in the lateral division of the central amygdala which results in inhibition of a subpopulation of peripherally projecting neurons in the medial division of the central amygdala (CeM). Common anxiolytics, such as diazepam, act as allosteric modulators of GABA(A) receptors. Because oxytocin and diazepam act on GABAergic transmission, it is possible that oxytocin can potentiate the inhibitory effects of diazepam if both exert their pre, – respectively postsynaptic effects on the same inhibitory circuit in the central amygdala. We found that in CeM neurons in which diazepam increased the inhibitory postsynaptic current (IPSC) decay time, TGOT (a specific oxytocin receptor agonist) increased IPSC frequency. Combined application of diazepam and TGOT resulted in generation of IPSCs with increased frequency, decay times as well as amplitudes. While individual saturating concentrations of TGOT and diazepam each decreased spontaneous spiking frequency of CeM neurons to similar extent, co-application of the two was still able to cause a significantly larger decrease. These findings show that oxytocin and diazepam act on different components of the same GABAergic circuit in the central amygdala and that oxytocin can facilitate diazepam effects when used in combination. This raises the possibility that neuropeptides could be clinically used in combination with currently used anxiolytic treatments to improve their therapeutic efficacy.



## 1. Introduction

Oxytocin is a nonapeptide which has been shown to have anxiolytic properties (see Table 1) and may have potential for therapeutic use. Application of exogenous oxytocin affects mother–infant interactions (Nelson and Panksepp, 1996; Pedersen and Prange, 1979), facilitates pair bonding (Bales et al., 2007), affects maternal aggression (Consiglio et al., 2005) and, most importantly, exerts an anxiolytic effect in various behavioral paradigms (Bale et al., 2001; Blume et al., 2008; Ring et al., 2006; Uvnas-Moberg, 1994; Windle et al., 1997, see also Table 1). In a number of these cases a role for endogenous oxytocin has been established, notably for maternal behavior (Van Leengoed et al., 1987), maternal aggression (Bosch et al., 2005) and for anxiolysis (Neumann et al., 2000; Waldherr and Neumann, 2007). Recent studies have shown oxytocin effects on human emotional behavior: in particular, oxytocin increases trust (Kosfeld et al., 2005; Baumgartner et al., 2008), reduces amygdala activation upon exposure to aversive stimuli (Kirsch et al., 2005; Singer et al., 2008), and attenuates affective evaluations of conditioned faces (Petrovic et al., 2008). At the circuit level, we and others (Huber et al., 2005; Terenzi and Ingram, 2005) have shown that oxytocin exerts a direct excitatory effect on GABAergic neurons in the lateral division of the central amygdala (CeL, Paxinos and Watson, 1998), resulting in an inhibition of target neurons in the medial division of the central amygdala (CeM, Paxinos and Watson, 1998) and may thereby decrease anxiety and fear responses.

Anxiety disorders are commonly treated with benzodiazepines such as diazepam and lorazepam. Though efficient, side effects include drowsiness and amnesia and long-term use may lead to dependence. Multiple studies have demonstrated that intra-amygdala injections of benzodiazepines produce anxiolytic effects as measured by inhibitory avoidance of open arms on the elevated T-maze (Graeff et al., 1993; Pesold and Treit, 1995), social interaction test (Gonzalez et al., 1996), defensive freezing (Helmstetter, 1993), conditioned avoidance (Krysiak et al., 2000) and conflict

* Corresponding author at: Centre for Psychiatric Neurosciences, Department of Psychiatry, University Hospital Center Lausanne (CHUV), Prilly-Lausanne, Switzerland. Tel.: +41 21 6436954; fax: +41 21 6436950.
*E-mail address:* ron.stoop@unil.ch (R. Stoop).

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**Table 1**
Studies where diazepam and oxytocin were found to exert similar effects on anxiety as assessed in different behavioral paradigms.

| Paradigm | Oxytocin | Diazepam |
|---|---|---|
| Open-field | Petersson et al., 1999; Veenema et al., 2007 | Depino et al., 2008 |
| Elevated-plus maze | Amico et al., 2004, 2008; Mantella et al., 2003; Windle et al., 1997; Veenema et al., 2007 | Depino et al., 2008; Wilson and Junor, 2008; Jaszberenyi et al., 2007; Burghardt and Wilson, 2006 |
| Elevated-zero maze | Ring et al., 2006 | Shepherd et al., 1994 |
| Light–dark box | Waldherr and Neumann, 2007 | Crawley and Goodwin, 1980; Chaouloff et al., 1997 |
| Novel object | Veenema et al., 2007 | Depino et al., 2008 |
| Fear conditioning | McCarthy et al., 1996 | Resstel et al., 2006; Brignell and Curran, 2006 |
| Novel environment | Amico et al., 2004 | Amikishieva and Semendyaeva, 2005 |
| Noise stress | Windle et al., 1997 | Breschi et al., 1995 |

situations (Nagy et al., 1979). Diazepam binds to GABA(A) receptors of the general form $\alpha_{(1\text{-}3or5)}\beta_{(1\text{-}3)}\gamma_{(2)}$ and allosterically enhances their function (Pritchett and Seeburg, 1990; Hadingham et al., 1993; Luddens and Korpi, 1995). As summarized in Table 1, diazepam and oxytocin have been shown to exert anxiolytic effects in the same experimental paradigms, although they have never been used in combination in a single study. Because oxytocin and diazepam both affect GABAergic transmission in the amygdala, yet at different levels (oxytocin pre-synaptically, diazepam postsynaptically), it is possible that, by acting on the same GABAergic circuit, oxytocin application could facilitate the effects of diazepam. To verify this hypothesis, we investigated in an in vitro preparation of the rat amygdala whether a combined application of diazepam and oxytocin would produce additive effects on the inhibition of central amygdala neurons.

## 2. Materials and methods

### 2.1. Animals

Animals were preweanling Sprague–Dawley OFA (Oncins France Strain A, from Charles River Laboratories, France) rats. Animals were 3–4 weeks old for optimized visualization of brain slices with infrared light (see below). A total of 28 animals were used of either sex. All animal experiments have been carried out according to the guidelines and regulation of the Swiss federal law on animal welfare.

### 2.2. Horizontal brain slices preparation

Rats were rapidly anesthetized with isoflurane and subsequently decapitated. The brain was removed and immediately immersed in ice-chilled artificial cerebrospinal fluid (ACSF) composed of (in mM): 118 NaCl, 25 $NaHCO_3$, 10 D-Glucose, 2 KCl, 2 $MgCl_2$, 1.2 $NaH_2PO_4$, 2 $CaCl_2$. During the preparation of the slices, $MgCl_2$ concentration was increased to 10 mM. Brains were split and one hemisphere glued (cyanoacrylate glue ERGO, 1200) onto a cutting stage. The tissue was embedded with 2% agarose and cut on a vibroslicer (Compresstome™ VF-300, Precisionary Instruments, Greenville, NC) in 350 μm slices, that were transferred to an interface chamber (Fine Science Tools, Heidelberg, Germany). They were placed on a nylon grid at the surface of constant flowing (1.5 ml/min) ACSF, kept at room temperature and saturated with oxycarbon gas (95% $O_2$/5% $CO_2$) at pH 7.4. The slices were left for 1 h under these conditions before electrophysiological recordings started.

### 2.3. Electrophysiology

Recorded neurons were located in the CeM (Fig. 3c). Patch-clamp pipettes were pulled from hard borosilicate capillary glass using a horizontal puller (Sutter Instruments, Novato, CA, USA) in a multi-stage process. The resistance of the pipettes was 3–5 MΩ when filled with a KCl containing intracellular solution. The composition of this solution was (in mM): 150 KCl, 10 HEPES, 2 $MgCl_2$, 0.1 $CaCl_2$, 0.1 BAPTA, 2 ATP Na salt, 0.3 GTP Na salt. The final solution was adjusted at pH 7.22 and osmolarity of 292 mOsm.

IPSCs were measured by whole-cell patch-clamp method. Immediately before starting recordings, the blocker of AMPA receptor 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX) (10 μM) was added to the ACSF, to isolate IPSCs. The cells were approached under visual control by the use of infrared differential interference contrast video microscopy. After the formation of a GΩ seal between the tip of the pipette and the neuron, a more negative pressure was applied to rupture the membrane, thus establishing a whole-cell mode. The adjustment of capacitance compensation and series resistance compensation was done before recording the membrane currents. The holding potential was clamped at −70 mV; signals were amplified by an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA).

Spontaneous firing of single neurons was assessed in cell-attached configuration. In order to generate spontaneous activity in the central amygdala, slices were perfused with low $Ca^{2+}$ ACSF (in mM: 118 NaCl, 25 $NaHCO_3$, 10 glucose, 5 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, and 1.2 $NaH_2PO_4$) to enhance spontaneous activity. The glass recording electrodes were filled with the same KCl containing solution used for the whole-cell recordings.

### 2.4. Drugs and chemicals

To evaluate the effects of oxytocin, we used the specific oxytocin receptor agonist [Thr4,Gly7]-oxytocin (TGOT), diluted in ACSF at a final concentration of 0.4 μM (Huber et al., 2005; Terenzi and Ingram, 2005). Since in this study it was the only agonist used for oxytocin receptor activation, it is sometimes referred to directly as "oxytocin". Diazepam (Roche, Basel, CH) was diluted in ACSF to obtain a final concentration of 10 μM (Rudolph et al., 1999; Marowsky et al., 2004). The drugs were bath-perfused according to the protocol summarized in Figs. 1a and 2a. NBQX was perfused throughout the recording session. After 5 min stabilization, TGOT was added to the perfusion bath during 30 s, followed by 10 min washout. Diazepam was subsequently perfused for a period of 10 min to allow its effects to reach a plateau level (see Kang-Park et al., 2004), followed by a novel TGOT application (30 s).

### 2.5. Data acquisition and analysis

Electrophysiological data were acquired via a Digidata 1200 interface (Axon Instruments, Foster City, CA, USA) and an IBM-compatible computer running pClamp 9.2 (Axon Instruments). Patch-clamp signals were filtered at 5 kHz, digitized at a rate of 10 kHz, and amplified 20 times. Frequency, amplitude, rise and decay times of GABAergic inhibitory postsynaptic currents (IPSCs) were analyzed with MiniAnalysis 6.0.3 (Synaptosoft, Decatur, GA, USA). Decay time was defined as the time required for the currents to reach 80% of the resting state after the IPSC peak. Spiking frequency recorded by cell-attached patch clamp was assessed over 30 s periods. Values in the text represent mean ± standard error of the mean. For the statistical analysis the paired Student's *t*-test was applied.

## 3. Results

### 3.1. Individual and combined effects of oxytocin and diazepam on IPSC characteristics in the CeM

The protocol of drug application that we used for this study was especially adapted to the perfusion of diazepam because of its lipophilic properties. Washout of diazepam necessitates thorough and extensive rinsing and it is for this reason that we decided for a protocol in which the effects of the oxytocin receptor agonist TGOT were always tested before application of diazepam was performed. Subsequent combined application of oxytocin and diazepam was performed immediately after the isolated effects of diazepam had been tested, without awaiting washout of diazepam itself (Fig. 1a).

The inserts below the protocol in Fig. 1 show typical responses that we obtained through whole-cell patch-clamp recordings from neurons in the CeM. The inward currents represent inhibitory postsynaptic currents that were obtained with a high concentration

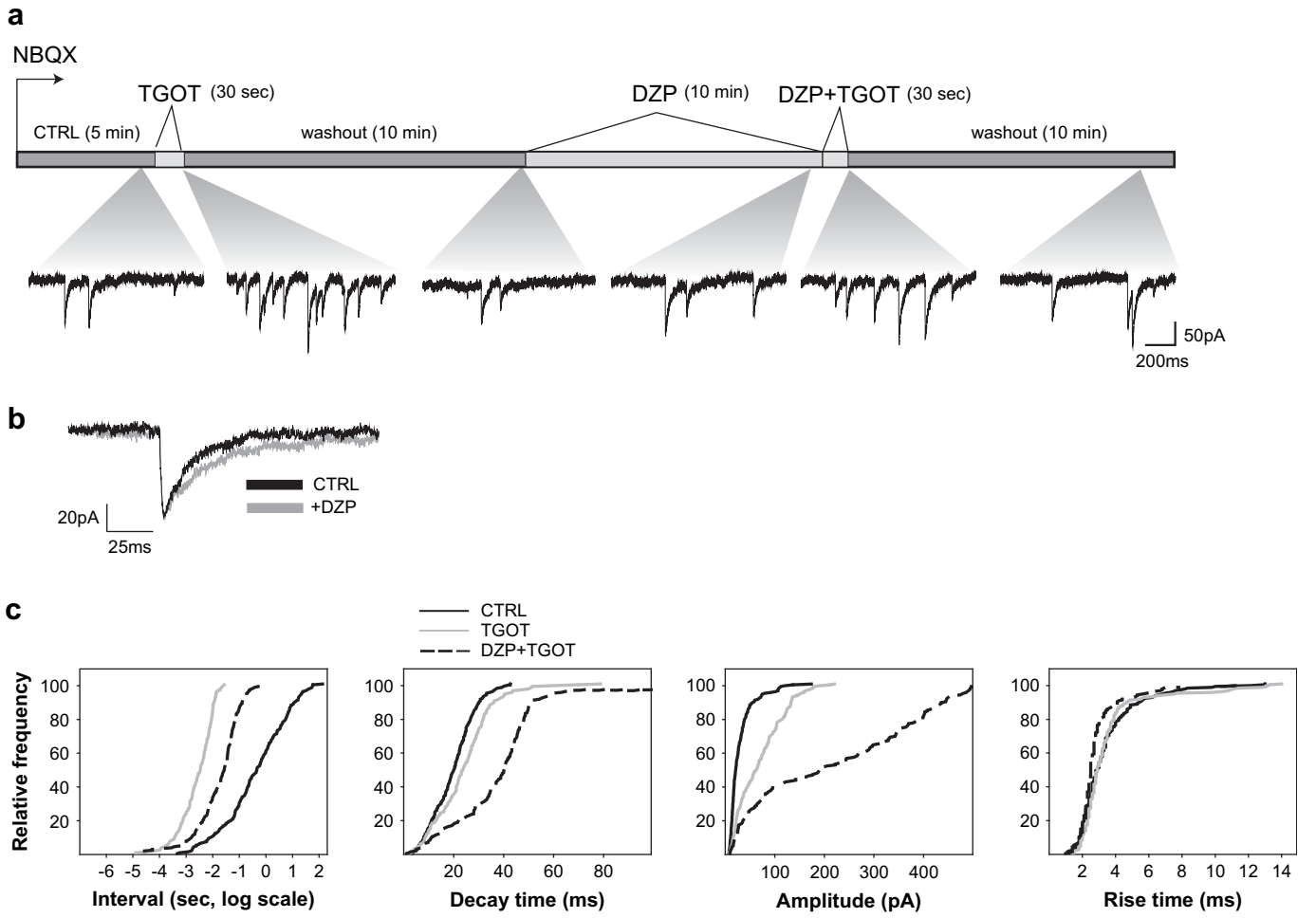


**Fig. 1.** Examples of IPSC traces recorded in the CeM and their cumulative distribution histograms following perfusion of oxytocin agonist TGOT, diazepam (DZP), and a combination of the two substances. (a) Time course of drug perfusion. Insets show representative traces of IPSCs recorded from a cell in the CeM at the times indicated. (b) Representative IPSC recorded under basal conditions overlapped with an IPSC recorded during diazepam, showing the increase in decay time. (c) Cumulative distribution histograms of different IPSC characteristics taken from the example trace shown in (a).

of KCl (150 mM) in the intracellular pipette solution and in the presence of the AMPA receptor blocker NBQX. In several instances we verified, by application of bicuculline, that NMDA currents were not activated at the holding potential of −70 mV (bicuculline blocked all currents, data not shown). TGOT application caused an increase in IPSC frequency (Fig. 1c) and an ensuing decrease in interval as shown in the cumulative distribution histogram for this trace (Fig. 1c). Diazepam did not affect intervals, but caused an increase in decay times as can be seen through the overlapping traces of similar amplitude (Fig. 1b) and in the cumulative distribution histogram (Fig. 1c). In this example, TGOT caused an increase in frequency of currents with large amplitudes, diazepam application increased the amplitudes even more (Fig. 1c, third panel). TGOT and, in particular, diazepam effects on amplitudes were less pronounced in other recordings (see averages in Fig. 2). In this trace as well as in others, none of the applications affected the rise times (Fig. 1c, right panel).

### 3.2. Average effects of oxytocin and diazepam on IPSC characteristics

In total, we applied all steps of the above protocol to 21 neurons in the CeM that we were able to keep in whole-cell patch-clamp configuration throughout the whole protocol. For each series of applications a new slice was taken and quality of the whole-cell patch-clamp configuration was monitored throughout the recording by regular application of small voltage steps. Following TGOT application (0.4 μM), we observed on average increases in IPSC frequencies from 0.9 ± 0.2 Hz to 3.64 ± 1 Hz ($p < 0.01$, $n = 21$), similar to what we had previously found (Huber et al., 2005). Increases in IPSC frequency were accompanied by increases in IPSC amplitude (42.5 ± 3 pA to 66.3 ± 8.3 pA, $p < 0.01$, $n = 21$). Washout of TGOT led within 10 min to a complete return of these values to levels before TGOT application (Figs. 1a and 2a). To neurons that had responded to TGOT, we subsequently applied diazepam (10 μM). Application of diazepam produced an increase in decay time (from 31.6 ± 1.9 to 36.4 ± 1.7 ms, $p < 0.01$, $n = 21$), but not in IPSC frequency (before: 1.05 ± 0.3 Hz; after diazepam: 1.18 ± 0.6 Hz; p = n.s.). Diazepam also seemed to increase IPSC amplitude, but this effect did not reach statistical significance (from 44.6 ± 2.8 pA to 55.3 ± 7 pA, $p = 0.1$, $n = 21$).

Neurons that had responded to TGOT and had been exposed to diazepam were subsequently exposed to a combination of 0.4 μM TGOT and 10 μM diazepam. TGOT again caused a significant increase in IPSC frequency (from 1.2 ± 0.6 to 2.7 ± 1.8 Hz, $p < 0.05$, $n = 21$). The combined application of TGOT and diazepam did not result in decay times significantly different from diazepam application alone (TGOT + DZP: 37.2 ± 2.1 ms vs. 36.4 ± 1.7 ms), but the

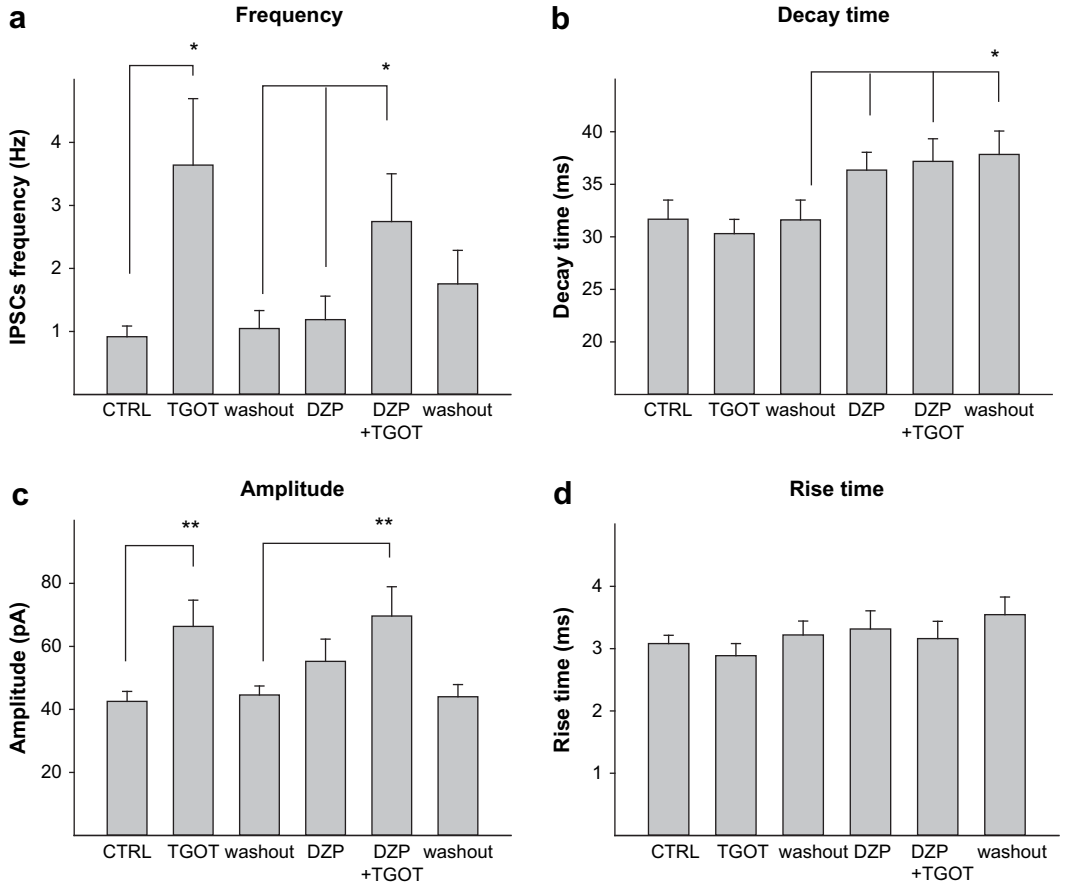


**Fig. 2.** Average changes in IPSC characteristics induced by TGOT and diazepam (DZP) (a) frequency, (b) decay time, (c) amplitude and (d) rise time. TGOT significantly increased IPSC frequency and amplitude when applied alone as well as when co-applied with diazepam. Diazepam induced a significant increase in decay time. Rise time was not affected by any treatment ($^{*}p < 0.05$, $^{**}p < 0.01$ $n = 21$).

amplitude now reached a significantly higher level than control (from 44.3 ± 2.9 pA to 69.7 ± 8.5, $p < 0.01$, $n = 21$), albeit not more than upon the application of TGOT alone. To summarize, co-application of diazepam and TGOT resulted in a combined effect of the two compounds, namely, an increase in frequency and amplitude of IPSCs, and a prolongation in decay time. None of the applied drugs affected on average the rise times of the IPSCs (Fig. 2d).

### 3.3. Oxytocin facilitates the inhibitory effects of diazepam on spontaneous spiking activity in CeM

Above findings show (1) that diazepam and oxytocin affect different characteristics of inhibitory postsynaptic currents and (2) that these effects can occur in the same neurons of the CeM. The question therefore arises whether their combined application may lead to stronger inhibition of spontaneous activity in individual CeM neurons. To understand how the excitability of CeM neurons was affected, we investigated spontaneous spiking activity of TGOT-inhibited neurons in cell-attached configuration.

As can be seen in Fig. 3a and b, application of TGOT during 30 s resulted in a modest, yet significant decrease in spontaneous spiking frequency (from 2.9 ± 0.3 to 2.5 ± 0.3 Hz, $n = 11$, $p < 0.05$). Washout of TGOT (10 min) with ACSF completely reestablished spiking frequency to initial levels. Diazepam was then applied during 10 min, resulting in a decrease in spiking activity from 3.2 ± 0.4 Hz to 2.4 ± 0.4 Hz ($p < 0.01$, $n = 11$), which was comparable to the decrease following previous application of TGOT. At this point, a second application of TGOT during 30 s produced a further significant decrease in spiking frequency, which dropped to 1.7 ± 0.3 Hz ($p < 0.01$, $n = 11$). After 10 min, following complete washout of the oxytocin agonist, the spontaneous spiking frequency settled back to 2.2 ± 0.5 Hz. This value was similar to the one obtained after application of diazepam, suggesting a long lasting effect of the benzodiazepine.

## 4. Discussion

The above whole-cell patch-clamp recordings show that oxytocin and diazepam have different effects on neurons in the CeM: namely, while diazepam mainly affects decay time, oxytocin affects frequency and amplitude of IPSCs and co-application results in a combined effect of the two substances. As to the effects of oxytocin and diazepam on spontaneous spiking, both substances induce a similar reduction in activity, which even further decreases when the two substances are applied together.

Thus, as illustrated in Fig. 3c, these findings support our previous model which proposed that inhibition of CeM neurons by oxytocin is brought about by synaptically released GABA, which most likely originates from neurons in the central lateral amygdala (Huber et al., 2005). Furthermore, they show that inhibition of CeM neurons, induced by application of diazepam, is facilitated by oxytocin-enhanced release of GABA. A second relevant aspect of this study is the evidence that the effects of oxytocin on CeM neurons are far shorter lasting than those of diazepam. This

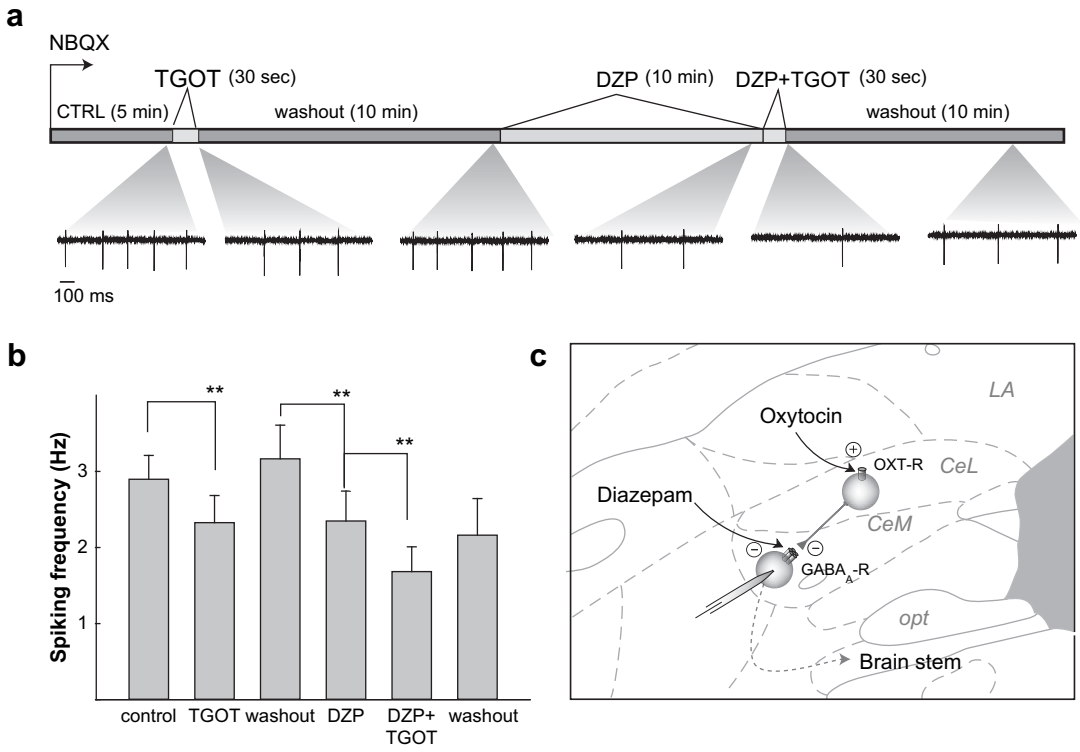


**Fig. 3.** Changes in spontaneous spiking activity in CeM neurons following perfusion with TGOT, diazepam (DZP) and their combination. (a) Time course of drug perfusion. Insets show 1 s excerpts of a representative recording before, during and after the application of the two substances. (b) Average spiking frequencies of CeM neurons upon stimulation with TGOT and diazepam. TGOT and diazepam application significantly decreased spontaneous spiking frequency. Co-application of diazepam and TGOT resulted in a significantly stronger inhibition of the spiking frequency as compared to diazepam and TGOT alone ($^{*}p < 0.05$, $^{**}p < 0.01$, $n = 11$). (c) Schematic drawing illustrating the sites of the recordings, notably in the CeM, and the modulatory effects of oxytocin and diazepam ((+) = excitation; (−) = inhibition). Oxytocin exerts a direct excitatory effect through its receptor (OXT-R) on CeL neurons that subsequently produce GABAergic inhibition of CeM neurons. Diazepam inhibits CeM neurons acting directly on the GABA(A) receptor ($GABA_A$-R) (LA = Lateral amygdaloid nucleus; opt = optic tract). Adapted from Paxinos and Watson (1998).

suggests that oxytocin is capable of triggering GABA release in an acute and rapid fashion, while diazepam, due to its binding kinetics to the GABA receptor or due to its lipophilic properties, leads to a longer lasting inhibition.

Intra-amygdaloid application of benzodiazepines is known to produce anxiolysis (Nagy et al., 1979) via a specific inhibition of central amygdala neurons (Beck and Fibiger, 1995). Thus, the reduced spiking activity observed upon application of diazepam should reflect at least in part its anxiolytic properties. The observation that application of oxytocin resulted in a similar reduction in spiking frequency is in keeping with a number of reports about anxiolytic properties of oxytocin. Of major relevance is the finding that simultaneous application of oxytocin with diazepam facilitated the inhibitory effects of diazepam. The concentrations of both the oxytocin agonist TGOT and diazepam used in our experiments were actually such to attain maximal effects on IPSC characteristics. Indeed, diazepam is known to saturate its binding sites in the amygdala at concentrations of 1 μM (Rudolph et al., 1999; Marowsky et al., 2004), while TGOT produces similar increases in IPSC frequency at a range of concentrations starting from 0.2 μM up to 1 μM. The concentration of 0.4 μM was chosen to assure maximal effects without desensitization (Huber et al., 2005; Terenzi and Ingram, 2005). The additive effects of TGOT and diazepam raise the possibility of obtaining stronger anxiolysis by combining the two substances. In addition, by virtue of its different site of action, oxytocin promises to be therapeutically effective in those circumstances in which chronic use of benzodiazepines has led to tolerance.

The above findings can be represented in the model as shown in Fig. 3c. Diazepam may affect local inhibition by direct effects on the GABA(A) receptor in the CeM. A number of mechanisms have been proposed how diazepam modulates GABAergic neurotransmission, including changes in single channel conductance (Eghbali et al., 1997), increased binding affinity of GABA for the receptor (Lavoie and Twyman, 1996; Krampfl et al., 1998), or modulation of GABA(A)R channel gating and direct effects on channel open transitions (Campo-Soria et al., 2006). In the hippocampal CA1 region, diazepam has been shown to increase the baseline holding current through a mechanism that could be blocked by picrotoxin, i.e. that resulted from a tonic release of GABA. This holding current appeared to be mediated by extrasynaptically expressed alpha-5 subunit containing GABA(A) receptors that are activated by low ambient GABA concentrations (Prenosil et al., 2006). While it is not the aim of the present study to distinguish between these different models, we did not find any evidence for direct effects of diazepam on GABAergic holding currents (data not shown). It is possible that the lower expression of alpha 5 subunits in the central amygdala (Fujimura et al., 2005) compared to the CA1 region (Pirker et al., 2000) could explain the absence of this effect of diazepam in our recordings.

It thus appears that diazepam effects in the amygdala require a phasic basic release of GABA. Is this phasic release caused by oxytocin that is endogenously present in the CeL? In the past, we have tested whether the application of an oxytocin receptor antagonist affects basic inhibition of neurons in the CeM (presumably by decreasing excitation in the CeL) but this did not seem to be the case (Huber et al., 2005). It thus seems that endogenous oxytocin, at least in our slice preparation, does not affect the basic effects of diazepam. However, this does not exclude that endogenous released oxytocin may affect diazepam responses

in vivo. In fact, our findings open the interesting possibility that increased release of endogenous oxytocin as observed during stress experiences (Ebner et al., 2005) could facilitate the inhibitory effects of diazepam.

Also because of their different sites of action, oxytocin and diazepam result in slightly different effects on IPSCs, namely, frequency and amplitude vs. decay time. Whether these differences can result in substance-specific differences in anxiolysis remains a matter for future investigations. To our knowledge, simultaneous administration of both substances in vivo in order to assess whether oxytocin facilitates anxiolytic effects of diazepam has not yet been thoroughly explored. Our study clearly shows that diazepam will be more effective, owing to increased IPSC frequency induced by oxytocin, and thereby provides the rationale to investigate the additive action of oxytocin and benzodiazepines in in vivo models of anxiety.

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