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Title: Endogenous 5-HT_{2B} receptor activation regulates neonatal respiratory activity *in vitro*

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Abstract
An implication of 5-HT_{2B} receptors in central nervous system has not yet been clearly elucidated. We studied the role of different 5-HT_2 receptor subtypes in the medullary breathing center, the pre-Bötzinger complex, and on hypoglossal motoneurons in rhythmically active transversal slice preparations of neonatal rats and mice. Local microinjection of 5-HT_2 receptor agonists revealed tonic excitation of hypoglossal motoneurons. Excitatory effects of the 5-HT_{2B} receptor agonist BW 723C86 could be blocked by bath application of LY 272015, a highly selective 5-HT_{2B} receptor antagonist. Excitatory effects of the 5-$HT_{2A/B/C}$ receptor agonist α-methyl-5-HT could be blocked by the preferential 5-HT_{2A} receptor antagonist ketanserin. Therefore, 5-HT induced excitation of hypoglossal motoneurons is mediated by convergent activation of 5-HT_{2A} and $_{2B}$ receptors. Local microinjection of BW 723C86 in the pre-Bötzinger complex increased respiratory frequency. Bath application of LY 272015 blocked respiratory activity, whereas ketanserin had no effect. Therefore, endogenous 5-HT appears to support tonic action on respiratory rhythm generation via 5-HT_{2B} receptors. In preparations of 5-HT_{2B} receptor deficient mice, respiratory activity appeared not altered. Whereas BW 723C86 and LY 272015 had no effects, bath application of ketanserin disturbed and blocked rhythmic activity. This demonstrates a stimulatory role of endogenous 5-HT_{2B} receptor activation at the pre-Bötzinger complex and hypoglossal motoneurons that can be taken up by 5-HT_{2A} receptors in the absence of 5-HT_{2B} receptors. The presence of functional 5-HT_{2B} receptors in the neonatal medullary breathing center indicates a potential convergent regulatory role of 5-HT_{2B} and 5-HT_{2A} receptors on the central respiratory network.

Introduction

5-HT modulates the respiratory rhythm in both neonate and adult mammals (Richter et al., 2003). Medullary respiratory regions are innervated by serotonergic neurons of the caudal brainstem raphe nuclei (Richerson, 2004). Local effects of 5-HT are mediated primarily by postsynaptic 5-HT receptors on respiratory interneurons and motoneurons. Besides the 5-HT_2 receptor subfamily these include 5-$HT_{1A,}$ 5-$HT_{4(a)}$ and possibly 5-HT_7 receptors (Manzke et al., 2003; Richter et al., 2003). In vivo studies have elucidated the effects of 5-HT_{1A} and 5-HT_2 receptor activation on medullary respiratory neurons (Lalley et al., 1994; Lalley et al., 1995). 5-HT accelerates rhythmic nerve activity in the isolated brainstem-spinal cord preparation of neonatal rats, in which respiratory network functions are preserved (Morin et al., 1990; Morin et al., 1991, 1992; Di Pasquale et al., 1994; Onimaru et al., 1998; Ballanyi et al., 1999). These findings were substantiated in respiratory active slice preparations that included the essential part of the respiratory network, i.e. the pre-Bötzinger complex (PBC) (Al-Zubaidy et al., 1996; Johnson et al., 1996). Intracellular recordings in such preparations revealed postsynaptic 5-HT_{1A} and 5-HT_2 receptors on respiratory interneurons of the PBC (Schwarzacher et al., 2002).

During the late embryonic and postnatal phase, 5-HT appears to be stimulatory by depolarization of PBC interneurons and motoneurons. More complex 5-HT effects have been reported (Onimaru et al., 1998) including an initial stimulation and a subsequent inhibition of respiration. After all, a stimulatory role seems to be the prominent function of 5-HT in the developing respiratory network (Richerson, 2004). This stimulatory function can be mainly attributed to depolarization of respiratory neurons via postsynaptic 5-HT_2 receptors. So far, there is only limited information about the contribution of individual 5-HT_2 receptor subtypes (Pena and Ramirez, 2002; Schwarzacher et al., 2002).

The 5-HT_2 receptor subfamily includes 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, which are coupled to activation of phospholipase C (PLC) (Hoyer et al., 1994; Hoyer et al., 2002). The 5-HT_{2B} receptor has been cloned in mouse and human (Loric et al., 1992; Choi et al., 1994), and is an important regulator of embryonic development. 5-HT2B receptor inactivation leads to death in approximately 30% of embryonic, and an additional 30% of neonatal mice (Nebigil et al., 2000). However, evidence for a functional role of 5-HT_{2B} receptors within the central nervous system is sparse. So far, the 5-HT_{2B} receptor has been implicated in neurobiological mechanisms of anxiety, schizophrenia, autism, migraine and spreading depression (Baxter et al., 1994; Kennett et al., 1996).

We assessed the function of 5-HT_{2B} receptors in rhythmically active transversal slices of neonatal rats, a standard preparation, that has been frequently used in studies on serotonergic regulation of central respiratory control. The results from rat preparations were further substantiated in slice preparations of neonatal wild type and 5-HT_{2B} receptor deficient mice. We demonstrate, for the first time, a stimulatory role of 5-HT_{2B} receptors at both PBC and hypoglossal motonucleus that is abolished in 5-HT_{2B} receptor knockout mice and taken over by 5-HT_{2A} receptors. The presence of functional 5-HT_{2B} receptors in the neonatal medullary breathing center indicates a regulatory role of the 5-HT_{2B} receptor during development of the central respiratory network.

Material and Methods:

Animals and preparations

Experiments were performed on one to five day old Sprague-Dawley rats or mice (wild type: PAS 129 SVJ, Charles River, Wiga, Germany; 5-HT_{2B} receptor deficient mice, Zentrale Tierexperimentelle Einrichtung, Universität Göttingen, Germany). As 5-HT_{2B} receptor deficiency causes postnatal death in approximately 30% of newborn mice (Nebigil et al., 2000), only subjects were chosen for experiments, that did not show severe disturbances of body constitution. All experiments conformed to the international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering. Animals were anesthetized with ether and decerebrated. The neuraxis was isolated during immersion in artificial cerebrospinal fuid (ACSF) that contained (in mM) 118 NaCl; 3 KCl; 1.5 $CaCl_2$; 1 $MgCl_2$; 2.0 $NaHCO_3$; 1.2 NaH_2PO_4; and 35 D-glucose (pH adjusted to 7.4 by gassing with 95% O_2, 5% CO_2). After removal of the cerebellum and transverse sectioning at the ponto-medullary junction and rostral to the spinal C1 level, the brainstem was glued to a PVC block with the rostral side down. The block was transferred to a vibratome (Vibroslice 752M, Campden Instruments, Sileby, UK) and serial transverse sections (100 µm) were made starting at the caudal level. As judged by the cytoarchitectonic structures in the slices (Feldman et al., 1991; Smith et al., 1991; Funk et al., 1993), serial sectioning was stopped caudal to the pre-BötC (Smith et al., 1991). A single transverse slice (rostro-caudal diameter: 300-600 µm) containing the pre-BötC was cut and transferred to the recording chamber. The preparation was placed on a mesh covering a metal ring (height: 2.5 mm) and fixed with a threaded frame. After a time period of 30 min of superfusing the preparation with ACSF (27°C, flow rate 2.7 ml/min), the K^+ concentration was increased to a total of 8 mM for long-term maintenance of respiratory activity (Smith et al., 1991; Funk et al., 1993; Al-Zubaidy et al., 1996).

Drug application and microinjection

The following drugs were added to the superfusate: 5-hydroxytryptamine (5-HT), α-methyl-5-hydroxytryptamine (α-methyl-5-HT), ketanserin (3-(2-[4-(4-fluorobenzoyl)-1piperidinyl]ethyl)-2,4-[1H,3H]quinazolinedione), BW 723C86 (l-[5-(2-thienylmethoxy)-1H-3-indoyl] propan-2-amine hydrochloride, (Kennett et al., 1996), LY272015 (6-methyl-1,2,3,4-tetrahydro-1-[3,4-dimethoxyphenyl)methyl]-9*H*-pyrido[3,4-*b*] indole hydrochloride, (Russell et al., 2002). All chemicals were obtained from Sigma (Deisenhofen, Germany). In accordance with previous studies under similar experimental conditions (Morin et al., 1991, 1992; Di Pasquale et al., 1994; Al-Zubaidy et al., 1996; Schwarzacher et al. 2002), all drugs were bath applied in the range of 10-100 µM in ACSF. Initial tests revealed no principal differences in effects at concentrations of 10, 50, and 100 µM for α-methyl-5-HT and BW 723C86. Concentrations of 50 µM resulted in constant effects of 5-HT receptor agonists as well as full blockade of agonist effects by the corresponding 5-HT receptor antagonists (50 µM). For a good comparison of effects in different preparations, all drugs were bath applied at concentrations of 50 µM in ACSF, if not noted differently. The applied quantities of all agonists corresponded to the volume of the recording chamber (7.5 ml).

Local microinjection was used to determine the effects of different serotonin agonists on the respiratory system. The agonists were directly injected into the PBC or in the XII nucleus. The resulting reaction could be observed via extracellular recording of the inspiratory discharge. The microinjection pipettes were pulled from thin borsilicate glass capillaries (GC150 TF, Clark electromedical Instruments, UK) using a horizontal puller (Zeitz, Augsburg, Germany) and breaking the tip. Only pipettes with an average tip diameter of 5-10

μm were used. Injection quantities were measured by monitoring the meniscus of the solution with the aid of a stereo-microscope. To exclude artefacts due to mechanical disturbance of the tissue, control injections with ACSF were performed prior to every agonist injection. Injection quantities of less than 1.0 nl were chosen to minimise mechanical artefacts. In initial experiments, various concentrations (1-20 mM in ACSF) of α-methyl-5-HT and BW 723C86were tested. Injections in quantities of 0.4 pmol at concentrations of 10 mM in ACSF (0.4 nl α-methyl-5-HT or BW 723C86) turned out to be the minimal amount to produce significant responses and were therefore used in all further experiments.

Recordings

Inspiratory discharge of XII motoneurons was recorded extracellularly with suction electrodes applied to proximal ends of cut ventral XII nerve rootlets. Recorded signals were band pass filtered at 0.2-2.5 kHz and integrated with a time constant of 20-30 ms. During the experiments, signals were also displayed on a chart recorder (Yokogawa, Amersfort, the Netherlands). Signals were amplified and recorded on a Macintosh computer via a MacLab/4s digital converter (AD Instruments, Castle Hill, Australia). Effects of drugs on XII rootlet recordings were evaluated by measuring the maximal amplitude of summated spike activity, the frequency of rhythmic bursts, and the duration of effects. Changes of amplitudes were calculated as percentage increase against background activity between spontaneous bursts or before appearance of effects, respectively. Regularity of rhythmic bursts was obtained by calculating the irregularity score (*S*) with the following formula: S_n = 100 X ABS($P_n - P_{n-1}$)/P_{n-1}, where S_n is the score of the *n*th cycle, P_n is its period, P_{n-1} is the period of the preceding burst, and ABS is the absolute value (for details, see Telgkamp et al., 2002).

Results

5-HT_{2A} and 5-HT_{2B} receptor activation in rat rhythmic slices

Bath application of the 5-HT_2 receptor agonist α-methyl-5-HT induced a tonic excitation in XII rootlet recordings with an 470 +/- 177% increase in summated spike amplitude for a duration of 6.1 +/- 1.6 min, and an increase of rhythmic burst frequency of 62.7 +/- 17.8% (n = 5) (Fig. 1A). Similarly, bath application of the 5-HT_{2B} receptor selective agonist BW723C86 induced a tonic excitation with an 350 +/- 112% increase in summated spike amplitude for a duration of 3.61 +/- 1.7 min, accompanied by an increase of burst frequency by 89.7 +/- 34.0% (n = 9) (Fig. 1B). Both agonists mimicked the effects of 5-HT, which induced a tonic excitation and an increase of burst frequency (n = 3), as previously described for different rat and mice preparations (data not shown, see Schwarzacher et al., 2002). Bath application of the 5-HT_{2A} receptor preferential antagonist ketanserin did not significantly affect amplitude, frequency or regularity of spontaneous rhythmic spike activity even after prolonged application of more then 60 minutes (n = 8) (Fig. 2A, C). In contrast, bath application of the 5-HT_{2B} receptor selective antagonist LY272015 led to a significant reduction in the amplitude, frequency and regularity of rhythmic burst activity after 2 min, and a continuous decrease of burst amplitude, frequency and regularity until a complete blockade of spontaneous activity after 36.3 +/- 16.9 min (n = 12) (Fig. 2B). The quantification of the effects of LY272015 is shown in Figure 2D. Wash out of LY272015 was started 10 min after stop of spike activity and resulted in recovery of spontaneous rhythmic burst activity after the subsequent 6-10 min (mean 7.3 +/- 1.6, n = 6). These results indicate that activation of 5-HT_{2B} receptors is required for maintenance of rhythmic activity in rat slice preparations.

In order to test effects of specific 5-HT receptor agonists on different neuronal groups, we performed microinjections in the PBC and the XII nucleus in rhythmic slice preparations. Microinjection of the 5-HT_2 receptor agonist α-methyl-5-HT (0.4 pM) in the PBC induced an immediate and reversible increase of burst frequency (364.2 +/- 124.3%, duration of increased frequency 66.7 +/- 12.4s, n = 3), whereas no tonic excitation of XII rootlets was recorded (Fig. 3A). The effects on burst frequency could be completely blocked by 20 min bath application of the preferential 5-HT_{2A} receptor antagonist ketanserin (n = 3). Microinjection of the 5-HT_{2B} receptor agonist BW723C86 (0.4 pM) in the PBC induced an immediate and reversible increase of rhythmic burst frequency (395.1 +/- 131.9%, duration of increased frequency 62.5 +/- 13.0s, n = 4), whereas no tonic excitation of XII rootlets was recorded (Fig. 3B). The effect on burst frequency could be completely blocked by 20 min bath application of the 5-HT_{2B} receptor antagonist LY272015 (n = 4) (Fig. 3C).

Microinjection of the 5-HT_2 receptor agonist α-methyl-5-HT (0.4 pM) in the XII nucleus induced tonic excitation of XII rootlets, whereas no change in burst frequency was recorded (n = 12) (Fig. 4A). Tonic excitation could be blocked by 20 min bath application of the 5-HT_{2A} receptor preferential antagonist ketanserin (50 µM, n = 6) (Fig. 4B), whereas 20 min bath application of the 5-HT_{2B} receptor antagonist LY272015 (n = 7) did not affect tonic excitation by microinjection of α-methyl-5-HT in the XII nucleus (Fig. 4C). Microinjection of the 5-HT_{2B} receptor selective agonist BW723C86 in the XII nucleus induced tonic excitation of XII rootlets, whereas no change in burst frequency was recorded (n = 17) (Fig. 4D). Tonic excitation by microinjection of BW723C86 in the XII nucleus was not affected by 30 min bath application of ketanserin (50 µM, n = 6) (Fig. 4E), but could be blocked by 30 min bath application of LY272015 (n = 14) (Fig. 4F).

In summary, the microinjection of agonists and bath application of antagonists demonstrates a clear pharmacological differentiation between 5-HT_{2A} and 5-HT_{2B} receptors with the drugs used in this study, at least under the conditions of rhythmic slice preparations in the neonatal rat. Activation of both receptor subtypes leads to an increase of burst frequency in the PBC and to tonic excitation of XII motoneurons.

5-HT$_{2A}$ and 5-HT$_{2B}$ receptor activation in mice rhythmic slices

In order to judge the effects of serotonergic drugs on 5-HT$_{2B}$ receptors in mutant mice, we first tested the different compounds in wild type mice. Slice preparations of newborn wild type mice (500–800 µm, P1-P5, 129 PAS, Charles River) were processed under the same conditions as rat slices. XII rootlet recordings in wild type mice slices revealed spontaneous rhythmic activity with very similar frequency and regularity when compared to rat slices. In addition, burst frequency and regularity did not significantly vary during 60 min recordings in unperturbed preparations (for quantification, see Fig. 5A, B). Bath application of 5-HT induced tonic excitation in XII rootlets (maximal increase of summated spike amplitude 507 +/- 181%, duration 5.4 +/- 2.7 min, n = 3), and an increase of burst frequency of 47 +/- 28% (n = 3), as previously described for different rat and mice preparations. Bath application of α-methyl-5-HT induced a strong tonic excitation (maximal increase of summated spike amplitude 636 +/- 319%, duration 6.8 +/- 2.9 min, n=7) and an increase of burst frequency (77.6 +/- 29.9%, n = 7). Similar effects could be repeated with bath application of α-methyl-5-HT in the presence of LY272015 (tonic excitation amplitude 653 +/- 246, duration 8.2 +/- 4.1 min; increase of burst frequency 69.2% +/- 27.6%, n = 6). Bath application of BW723C86 induced tonic excitation with an 195 +/- 158% increase in summated spike amplitude for a duration of 6.6 +/- 3.0 min, accompanied by an increase of rhythmic burst frequency by 93 +/- 29.7% (n = 4). These effects were not affected in the presence of ketanserin (n = 3) but were completely blocked in the presence of LY272015 (n = 3). Ten minutes after wash out of LY272015 the effects of bath applied BW723C86 were restored (n = 3). Bath application of ketanserin did not affect rhythmic burst amplitude, frequency and regularity of spontaneous bursts even after prolonged application of more then 80 min (n = 4) (Fig. 6A, for quantification see Fig. 6E). An increase to 75µM ketanserin did also not induce any changes in XII rootlet activity (n = 2). In contrast, bath application of LY272015 led to a significant reduction of burst amplitude, frequency and regularity after 2 min, and a continuous decrease of burst amplitude and frequency, as well as an increase in irregularity, until a complete blockade of spontaneous activity after 9-64 min (n = 6) (Fig. 6C, for quantification see Fig. 6G). Bath application of 5-HT (n = 2), or α-methyl-5-HT (n = 3) 1 min after stop of spontaneous spike activity induced tonic excitation as well as rhythmic burst activity during continuous bath application of LY272015. After a time period of 10 min between stop of spike activity and wash out of LY272015 spontaneous rhythmic burst activity could be restored 6-8 min after wash out (mean 6.75 +/-0.83, n = 4).

Taken together, these results of serotonergic drugs on rhythmic burst activity of newborn wild type mice slices are comparable to the results obtained from newborn rat slices. They demonstrate a clear pharmacological differentiation between 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors in mice slices.

Effect of 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor agonists in 5-HT$_{2B}$ receptor deficient mice rhythmic slices

A total of 25 medullary slices from 5-HT$_{2B}$ receptor deficient mice (P1 – P5) was evaluated. Slices exhibited regular spontaneous burst activity of 9 –17 bursts/min (mean 11.5 +/- 3.1) (Fig. 5A, 6A). Although slices from 5-HT$_{2B}$ receptor deficient mice exhibited a higher variability in regularity, the irregularity score was not significantly altered compared to wild type mice and rat preparations (Fig. 5A). In addition, burst frequency and regularity did not significantly vary during 60min recordings in unperturbed preparations (for quantification, see Fig. 5C). Bath application of 5-HT induced tonic excitation in XII rootlets (maximal increase of summated spike amplitude 453 +/- 143%, duration 4.3 +/- 2.2 min, n = 3) and an increase in burst frequency of 60 +/- 25% (n = 3), comparable to wild type mice preparation (see above). Bath application of α-methyl-5-HT induced a strong tonic excitation (maximal

increase of summated spike amplitude 540 +/- 424%, duration 6.3 +/- 1.2 min, n = 6) and an increase of burst frequency (98 +/- 38%, n = 6). Again, these effects were comparable with data from wild type mice preparations. Bath application of BW723C86 had no effect in 5-HT_{2B} deficient mice (n = 4). In addition, local microinjection of BW723C86 in the XII nucleus did not exhibit any effects (n = 3), whereas successive microinjection of α-methyl-5-HT at the same location induced tonic excitation on spike activity in XII rootlet recordings (n = 3). These findings illustrate that the selective 5-HT_{2B} receptor agonist BW723C86 does not exert effects at other 5-HT receptors.

Accordingly, bath application of LY272015 had no significant effect on spontaneous burst activity in slices from deficient mice within a period of 60 min (n = 6) (Fig. 6D, for quantification see Fig. 6H), whereas slices of wild-type mice reacted with a decrease of burst amplitude, frequency and regularity within few minutes (Fig. 6C, for quantification see 6G). These results demonstrate that the antagonist LY272015 exerts its inhibitory effects on rhythmic activity selectively via 5-HT_{2B} receptors. Whereas bath application of 50-75 µM of ketanserin had minor effects on spontaneous spike activity and rhythmic burst activity in rat and wild types mice slices (Fig. 2A, 6A; see above), bath application of ketanserin led to severe disturbances of rhythmic activity within 2-10 min and a complete blockade of spike activity within 20 - 30 min in slices from 5-HT_{2B} receptor deficient mice (n = 5) (Fig. 6B). The quantification of the ketanserin effects in wild type mice and 5-HT_{2B} receptor deficient mice are shown in Figure 6E, F. In summary, in 5-HT_{2B} deficient mice, an endogenous 5-HT_{2A} receptor activity appears to maintain spontaneous rhythmic activity in respiratory centers.

Discussion

In this study, the presence of functionally active 5-HT_{2B} receptors was demonstrated in spontaneous rhythmically active medullary slice preparations of newborn rats and mice. Pharmacological activation of 5-HT_{2B} receptors led to tonic excitation of motoneurons as well as to an increase of rhythmic respiratory activity by stimulation of neurons in the PBC. Comparable effects were elicited with activation of 5-HT_{2A} receptors. These two 5-HT_2 receptor subtypes together appear to exert a crucial role in activation and maintenance of spontaneous respiratory activity within the given conditions of rhythmically active slice preparations. The inhibition of 5-HT_{2B} receptors by bath application of a selective 5-HT_{2B} receptor antagonist LY272015 resulted in a complete blockade of rhythmic respiratory activity. Genetic blockade of 5-HT_{2B} receptor could be compensated, as rhythmic respiratory activity was apparently unchanged in 5-HT_{2B} receptor deficient mice. However, application of the 5-HT_{2A} receptor antagonist ketanserin induced severe arrhythmia and/or complete blockade of respiratory activity in 5-HT_{2B} receptor deficient mice that did not induce detectable changes in respiratory activity of wild type preparations. Therefore, the genetic lack of 5-HT_{2B} receptors seems to be compensated by 5-HT_{2A} receptors.

Our report reveals for the first time that application of a 5-HT_{2B} receptor antagonist blocks respiratory activity under in vitro conditions. This supports previous observations, that endogenous 5-HT is required for respiratory rhythm generation at least under in vitro conditions (Al-Zubaidy et al., 1996; Pena and Ramirez, 2002). Serotonergic raphe neurons fire continuously (Richerson et al., 2001) and chemical stimulation of raphe neurons localized within the slice preparation leads to an activation of respiratory frequency that can be blocked by the pan-5-HT_2 receptor-antagonist methysergide (Al-Zubaidy et al., 1996). Furthermore, pharmacological blockade of 5-HT uptake leads to an increase of fictive respiratory activity in transversal slice and brainstem preparations (Morin et al., 1990; Morin et al., 1991; Di Pasquale et al., 1994; Pena and Ramirez, 2002). Schwarzacher et al. (2002) have shown that bath application and iontophoresis of the 5-HT_2 receptor agonist α-methyl-5-HT mimicked the effects of 5-HT, 5-HT-evoked tonic excitation of respiratory XII motoneurons is mediated by postsynaptic 5-HT_2 receptors and the excitatory effects on respiratory rhythm are also primarily attributable to postsynaptic 5-HT_2 receptors of PBC neurons. For a good comparison of effects in rats, wild type and mutant mice, all drugs were bath applied at concentrations of 50µM in ACSF in the present study. As initial tests revealed no principal differences in effects at concentrations of 10, 50, and 100 µM for α-methyl-5-HT and BW 723C86, no complete dose-response curves were obtained. Future work could clarify this issue. However, concentrations of 50 µM resulted in constant effects of 5-HT receptor agonists as well as full blockade of agonist effects by 50 µM of the corresponding 5-HT receptor antagonists.

An important issue of the present study is the specificity of the applied 5-HT receptor agonists and antagonists. As particularly the pan-5-HT_2 agonist α-methyl-5-HT has high affinity to all three receptors of the 5-HT_2 family, it has to be questioned if some of the effects of α-methyl-5-HT could be attributed to the activation of 5-HT_{2C} receptors. The effects of a selective 5-HT_{2B} agonist that could be completely blocked by the corresponding selective antagonist, which becomes inefficient in mutant 5-HT_{2B} mice, strongly support the conclusion that, under the conditions of our slice preparations, the observed effects on respiratory activity are mediated by 5-HT_{2B} but not 5-HT_{2C} receptors. So far, the 5-HT_{2B} receptor has been localized in discrete nuclei in cerebellum, lateral septum, dorsal hypothalamus and medial amygdala by immunocytochemistry (Duxon et al., 1997). In addition, 5-HT_{2B} receptor mRNA was detected by in situ hybridization in the hippocampus, the habenula, cortex, locus coeruleus, hypothalamus, cerebellum and several brainstem nuclei including raphe (Bonaventure et al., 2002). Moreover, the postnatal expression of 5-HT_{2C} receptors has not

been detected in these areas (Li et al., 2004). In addition, (Pena and Ramirez, 2002) did not find any affection of rhythmic activity by application of the 5-HT_{2C} receptor antagonist SB206533 in mice transversal slices.

All members of the 5-HT_2 receptor subfamily have the ability to activate PLC, which causes subsequent activation of protein kinase C (PKC) (Hoyer et al., 1994; Hoyer et al., 2002). Activation of PKC with the phorbol ester phorbol 12-myristate 13-acetate (PMA) could prevent depression of respiratory activity produced by 5-HT_2 antagonists (Pena and Ramirez, 2002). It appears likely that the endogenous activation of both 5-HT_{2A} and 5-HT_{2B} receptors affects common intracellular pathways. However, Tournois et al. (1998) showed also the existence of antagonistic transduction pathways for 5-HT_{2A} and 5-HT_{2B} receptors when endogenously coexpressed in the same cells. It is therefore possible that, in wild type mice, each receptor performs different tasks, but when 5-HT_{2B} receptors are eliminated the 5-HT_{2A} receptor has the ability to compensate this deficit only at places where both receptors are coexpressed.

Pena and Ramirez (2002) have attributed the endogenous effects of 5-HT to activation of 5-HT_{2A} receptors. In their report, the antagonists ketanserin 40 μM, piperidine 20 μM and spiperone 20 μM reduced fictive respiratory activity and action potential frequency in respiratory PBC neurons (Pena and Ramirez, 2002). We were unable to affect respiratory activity in wild type mouse and rat slice preparations, even at higher concentrations of ketanserin (50 to 75 μM). This discrepancy could eventually be explained by mice strain differences. In addition, a number of experimental conditions such as the rostrocaudal level and thickness of slices could vary between the different laboratories. However, this was observed only in wild type mice, whereas genetic deficiency of 5-HT_{2B} receptors leads to ketanserin-induced inhibitions of fictive respiration. Another explanation would be that 5-HT_{2B} receptors acts both at pre- (raphe neurons) and post-synaptic (PBC and XII neurons) levels with differential effects whereas 5-HT_{2A} receptors would have only postsynaptic actions. Nevertheless, endogenous activation of both 5-HT_{2A} and 5-HT_{2B} receptors appear to be important for respiratory rhythm generation in vitro.

Disturbances of breathing arising from failures of the medullary respiratory center may occur as consequences of pulmonary and cardiac diseases, hypoxia, head trauma, alcoholism and drug overdose among others (Richter et al., 2003). The 5-HT_{1A} receptor agonist buspirone was effectively used in the treatment of apneustic disturbances occurring after traumatic lesions of ponto-medullary regions (Wilken et al., 1997; Richter et al., 2003). Deficiency of the medullary serotonergic network has been hypothesized to be a potential cause of SIDS (Kinney et al., 2001). Significant decreases of 5-HT_{1A} and 5-HT_{2A} receptor immunoreactivity was reported in several brainstem areas including the ventrolateral medulla in SIDS patients (Ozawa and Okado, 2002). The 5-HT_{2B} receptor immunoreactivity remains to be evaluated in these patients. Serotonergic neurons in the medulla have recently been shown to be sensors of carbon dioxide and pH (Richerson, 2004). Therefore, serotonergic neurons could act as central chemoreceptors and important stimulators of respiratory rhythm. Our study reveals that 5-HT_{2B} receptor activation is essential for 5-HT stimulation of the neonatal medullary breathing center. The stimulatory effect of 5-HT on respiratory rhythm generation via a variety of 5-HT receptor subtypes and intracellular pathways opens new ways of pharmacological treatment.

References

Al-Zubaidy ZA, Erickson RL, Greer JJ (1996) Serotonergic and noradrenergic effects on respiratory neural discharge in the medullary slice preparation of neonatal rats. Pflugers Arch 431:942-949.

Ballanyi K, Onimaru H, Homma I (1999) Respiratory network function in the isolated brainstem-spinal cord of newborn rats. Prog Neurobiol 59:583-634.

Baxter GS, Murphy OE, Blackburn TP (1994) Further characterization of 5-hydroxytryptamine receptors (putative 5-HT2B) in rat stomach fundus longitudinal muscle. Br J Pharmacol 112:323-331.

Bonaventure P, Guo H, Tian B, Liu X, Bittner A, Roland B, Salunga R, Ma XJ, Kamme F, Meurers B, Bakker M, Jurzak M, Leysen JE, Erlander MG (2002) Nuclei and subnuclei gene expression profiling in mammalian brain. Brain Res 943:38-47.

Choi DS, Colas JF, Kellermann O, Loric S, Launay JM, Rosay P, Maroteaux L (1994) The mouse 5-HT2B receptor: possible involvement in trophic functions of serotonin. Cell Mol Biol (Noisy-le-grand) 40:403-411.

Di Pasquale E, Monteau R, Hilaire G (1994) Endogenous serotonin modulates the fetal respiratory rhythm: an in vitro study in the rat. Brain Res Dev Brain Res 80:222-232.

Duxon MS, Flanigan TP, Reavley AC, Baxter GS, Blackburn TP, Fone KC (1997) Evidence for expression of the 5-hydroxytryptamine-2B receptor protein in the rat central nervous system. Neuroscience 76:323-329.

Feldman JL, Smith JC, Liu G (1991) Respiratory pattern generation in mammals: in vitro en bloc analyses. Curr Opin Neurobiol 1:590-594.

Funk GD, Smith JC, Feldman JL (1993) Generation and transmission of respiratory oscillations in medullary slices: role of excitatory amino acids. J Neurophysiol 70:1497-1515.

Hoyer D, Hannon JP, Martin GR (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav 71:533-554.

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP (1994) International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol Rev 46:157-203.

Johnson SM, Smith JC, Feldman JL (1996) Modulation of respiratory rhythm in vitro: role of Gi/o protein-mediated mechanisms. J Appl Physiol 80:2120-2133.

Kennett GA, Bright F, Trail B, Baxter GS, Blackburn TP (1996) Effects of the 5-HT2B receptor agonist, BW 723C86, on three rat models of anxiety. Br J Pharmacol 117:1443-1448.

Kinney HC, Filiano JJ, White WF (2001) Medullary serotonergic network deficiency in the sudden infant death syndrome: review of a 15-year study of a single dataset. J Neuropathol Exp Neurol 60:228-247.

Lalley PM, Bischoff AM, Richter DW (1994) 5-HT-1A receptor-mediated modulation of medullary expiratory neurones in the cat. J Physiol 476:117-130.

Lalley PM, Bischoff AM, Schwarzacher SW, Richter DW (1995) 5-HT2 receptor-controlled modulation of medullary respiratory neurones in the cat. J Physiol 487 (Pt 3):653-661.

Li QH, Nakadate K, Tanaka-Nakadate S, Nakatsuka D, Cui Y, Watanabe Y (2004) Unique expression patterns of 5-HT2A and 5-HT2C receptors in the rat brain during postnatal development: Western blot and immunohistochemical analyses. J Comp Neurol 469:128-140.

Loric S, Launay JM, Colas JF, Maroteaux L (1992) New mouse 5-HT2-like receptor. Expression in brain, heart and intestine. FEBS Lett 312:203-207.

Manzke T, Guenther U, Ponimaskin EG, Haller M, Dutschmann M, Schwarzacher S, Richter DW (2003) 5-HT4(a) receptors avert opioid-induced breathing depression without loss of analgesia. Science 301:226-229.
Morin D, Monteau R, Hilaire G (1991) Serotonin and cervical respiratory motoneurones: intracellular study in the newborn rat brainstem-spinal cord preparation. Exp Brain Res 84:229-232.
Morin D, Monteau R, Hilaire G (1992) Compared effects of serotonin on cervical and hypoglossal inspiratory activities: an in vitro study in the newborn rat. J Physiol 451:605-629.
Morin D, Hennequin S, Monteau R, Hilaire G (1990) Serotonergic influences on central respiratory activity: an in vitro study in the newborn rat. Brain Res 535:281-287.
Nebigil CG, Choi DS, Dierich A, Hickel P, Le Meur M, Messaddeq N, Launay JM, Maroteaux L (2000) Serotonin 2B receptor is required for heart development. Proc Natl Acad Sci U S A 97:9508-9513.
Onimaru H, Shamoto A, Homma I (1998) Modulation of respiratory rhythm by 5-HT in the brainstem-spinal cord preparation from newborn rat. Pflugers Arch 435:485-494.
Ozawa Y, Okado N (2002) Alteration of serotonergic receptors in the brain stems of human patients with respiratory disorders. Neuropediatrics 33:142-149.
Pena F, Ramirez JM (2002) Endogenous activation of serotonin-2A receptors is required for respiratory rhythm generation in vitro. J Neurosci 22:11055-11064.
Richerson GB (2004) Serotonergic neurons as carbon dioxide sensors that maintain ph homeostasis. Nat Rev Neurosci 5:449-461.
Richerson GB, Wang W, Tiwari J, Bradley SR (2001) Chemosensitivity of serotonergic neurons in the rostral ventral medulla. Respir Physiol 129:175-189.
Richter DW, Manzke T, Wilken B, Ponimaskin E (2003) Serotonin receptors: guardians of stable breathing. Trends Mol Med 9:542-548.
Russell A, Banes A, Berlin H, Fink GD, Watts SW (2002) 5-Hydroxytryptamine(2B) receptor function is enhanced in the N(omega)-nitro-L-arginine hypertensive rat. J Pharmacol Exp Ther 303:179-187.
Schwarzacher SW, Pestean A, Gunther S, Ballanyi K (2002) Serotonergic modulation of respiratory motoneurons and interneurons in brainstem slices of perinatal rats. Neuroscience 115:1247-1259.
Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL (1991) Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. Science 254:726-729.
Wilken B, Lalley P, Bischoff AM, Christen HJ, Behnke J, Hanefeld F, Richter DW (1997) Treatment of apneustic respiratory disturbance with a serotonin-receptor agonist. J Pediatr 130:89-94.

Figure Legends

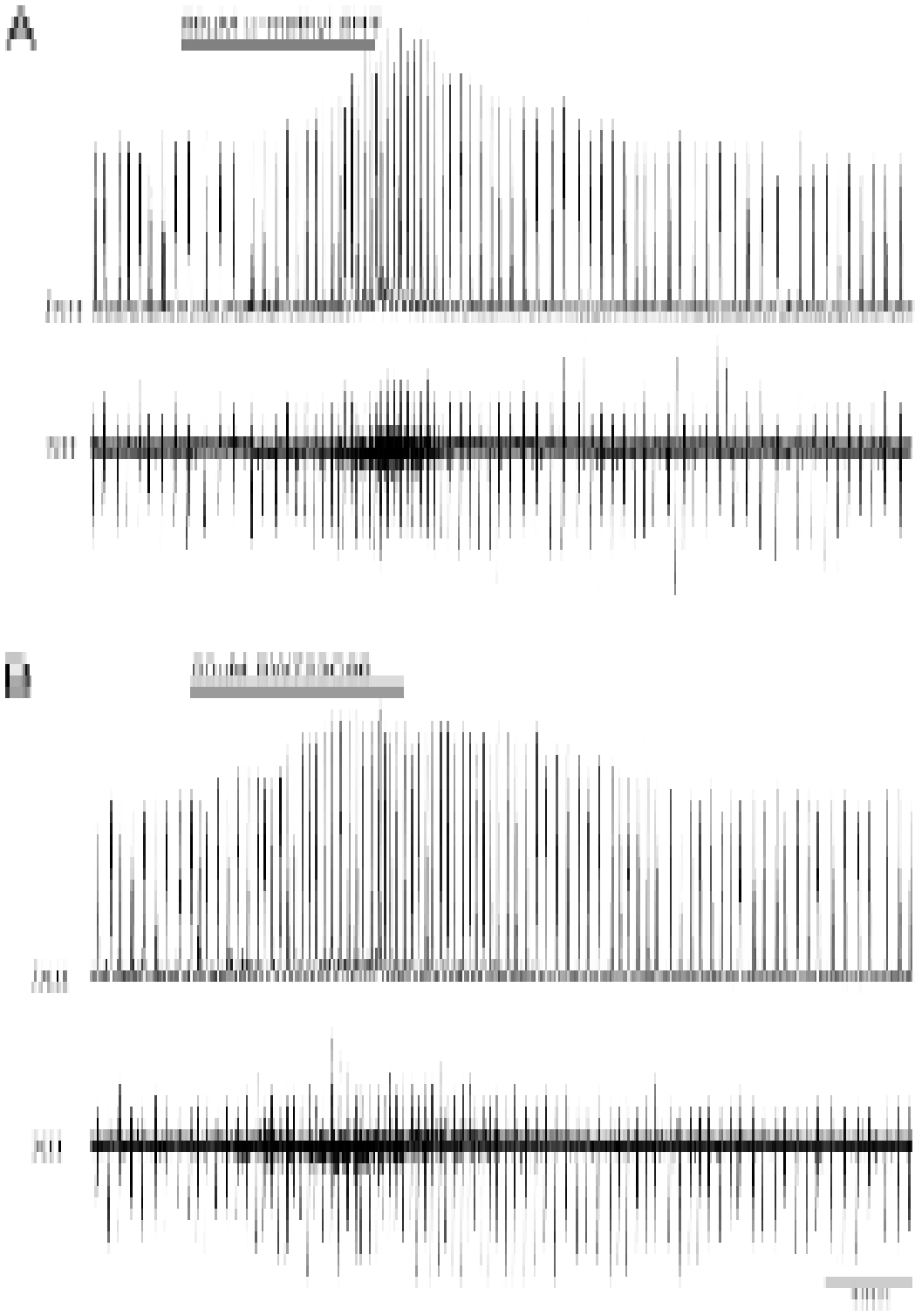

Figure 1
Transversal rhythmic slice preparations that contain the pre-Bötzinger complex were obtained from newborn rats. Spontaneous rhythmic respiratory activity was recorded from XII nerve rootlets (XII, ∫XII: integrated XII nerve rootlet recording).
A: Bath application of the 5-HT_{2A} receptor agonist α-methyl 5-HT led to an increase of respiratory burst frequency and to tonic spike discharge. B: Similar effects could be induced by bath application of the 5-HT_{2B} receptor agonist BW723C86.

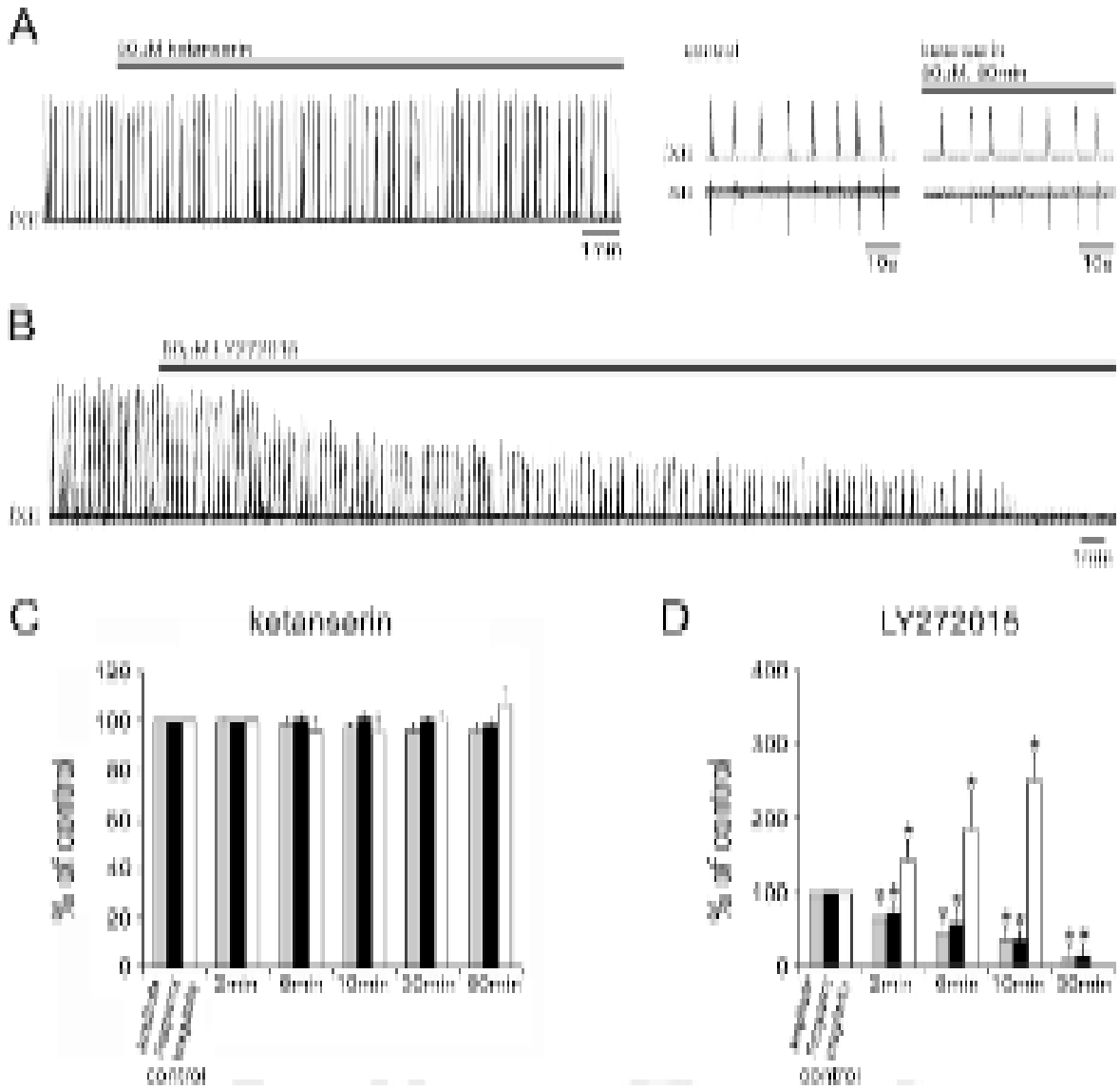

Figure 2
Bath application of the 5-HT_{2A} receptor antagonist ketanserin did not influence regular respiratory activity even after prolonged exposure in rat preparations (A). In contrast, bath application of the 5-HT_{2B} receptor antagonist LY272015 caused a gradual decrease of amplitude and frequency of respiratory bursts which led to a total stop of respiratory activity (B). C, D: Histograms plotting the percentage changes of rhythmic burst amplitude (grey columns), burst frequency (black columns) and irregularity (white columns) following bath application of the 5-HT_{2A} receptor antagonist ketanserin (C) and the 5-HT_{2B} receptor antagonist LY272015 (D). Significant changes were caused only after LY272015 application (Note the different scaling in D). *$p < 0.05$ (Student`s paired *t* test) compared with control.

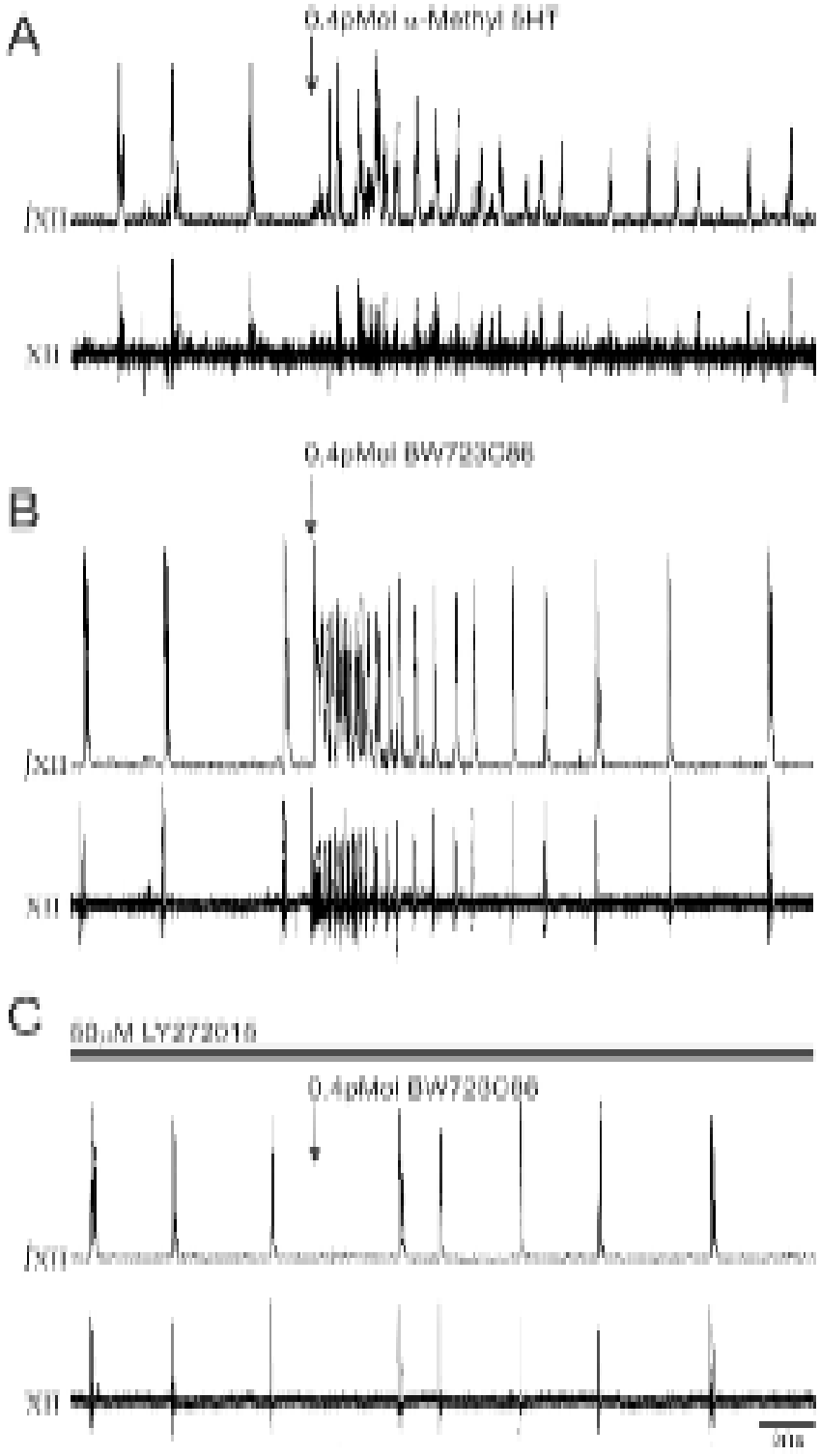

Figure 3
Local microinjection of the 5-HT_{2A} receptor agonist α-methyl-5-HT (A) or the 5-HT_{2B} receptor agonist BW723C86 (B) into the PBC induced a temporary activation of spike discharge and increase of rhythmic burst frequency in XII nerve rootlet recordings (∫XII; XII). C: During bath application of the 5-HT_{2B} receptor antagonist LY272015 (50 μM, 10 min) consecutive injection of BW723C86 exhibited no effect. B and C were obtained from the same slice preparation. Note that no tonic spike discharge was elicited by PBC microinjections.

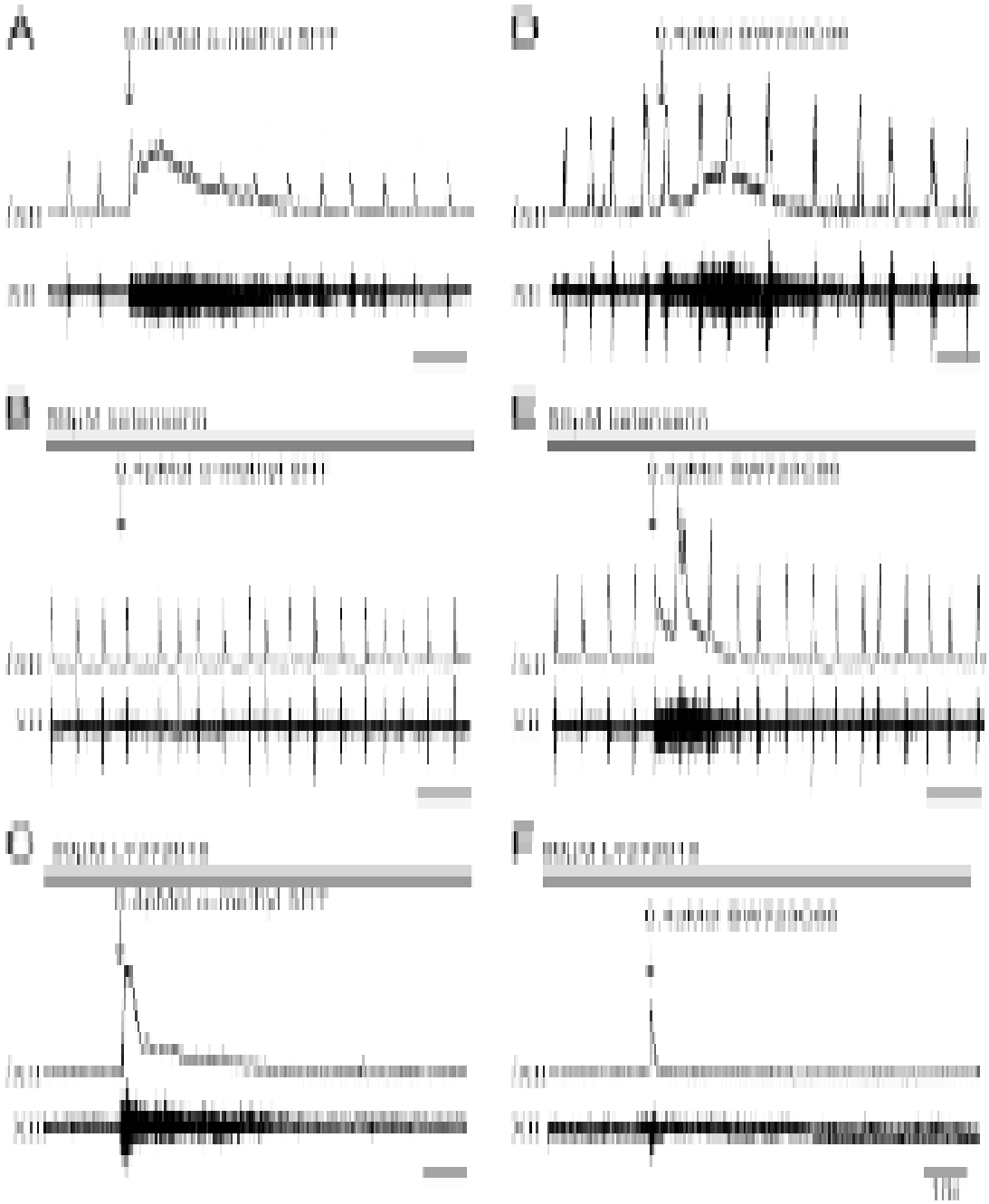

Figure 4
Functional localisation of 5-HT_{2B} and 5-HT_{2A} receptors on XII motoneurons by local microinjection of agonists into the XII nucleus and application of corresponding antagonists. Microinjection of the 5-HT_{2A} receptor agonist α-5-HT evoked tonic excitation of XII motoneurons (A) that could be inhibited by bath application of the 5-HT_{2A} receptor antagonist ketanserin (B). C: Bath application of the 5-HT_{2B} receptor antagonist L272015 failed to inhibit α-methyl-5-HT induced effects. D: Microinjection of the 5-HT_{2B} receptor agonist BW723C86 into the XII nucleus also generated tonic excitation of XII rootlets which was not altered by bath application of ketanserin (E), but could be inhibited by preincubation of the slice with LY272015 (F). Note that microinjections into the XII nucleus did not induce changes in respiratory frequency. Prolonged bath application of LY272015 led to stop of respiratory activity in C and F. A, B and E were obtained from the same slice preparation. C, D and F were obtained from another preparation.

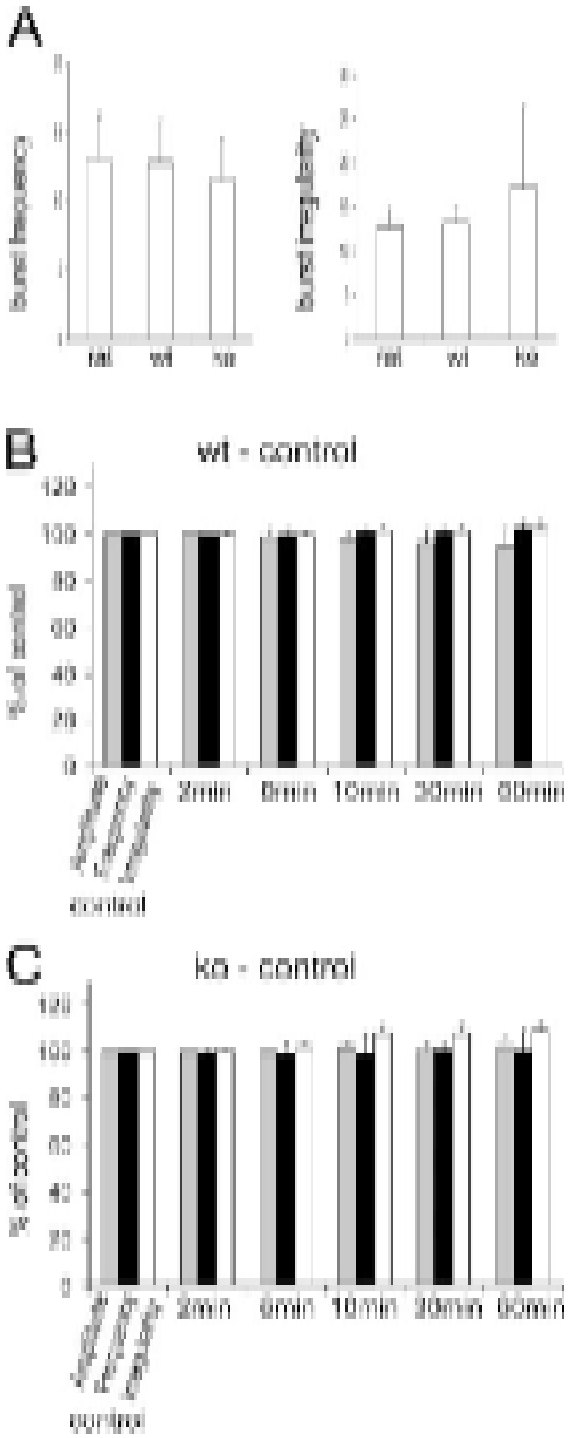

Figure 5
Comparison of spontaneous rhythmic activity in slice preparations from rat (n = 12), wild type mice (wt, n = 12), and 5-HT_{2B} receptor deficient mice (ko, n = 12). Burst frequency and irregularity was measured 30 min after start of hypoglossal rootlet recordings in unperturbed slices. No significant differences between animals could be observed (A). Burst amplitude, frequency and irregularity did not change significantly during 60 min of continuous registration in wild type mouse (wt, n = 6), and 5-HT_{2B} receptor deficient mice (ko, n = 6).

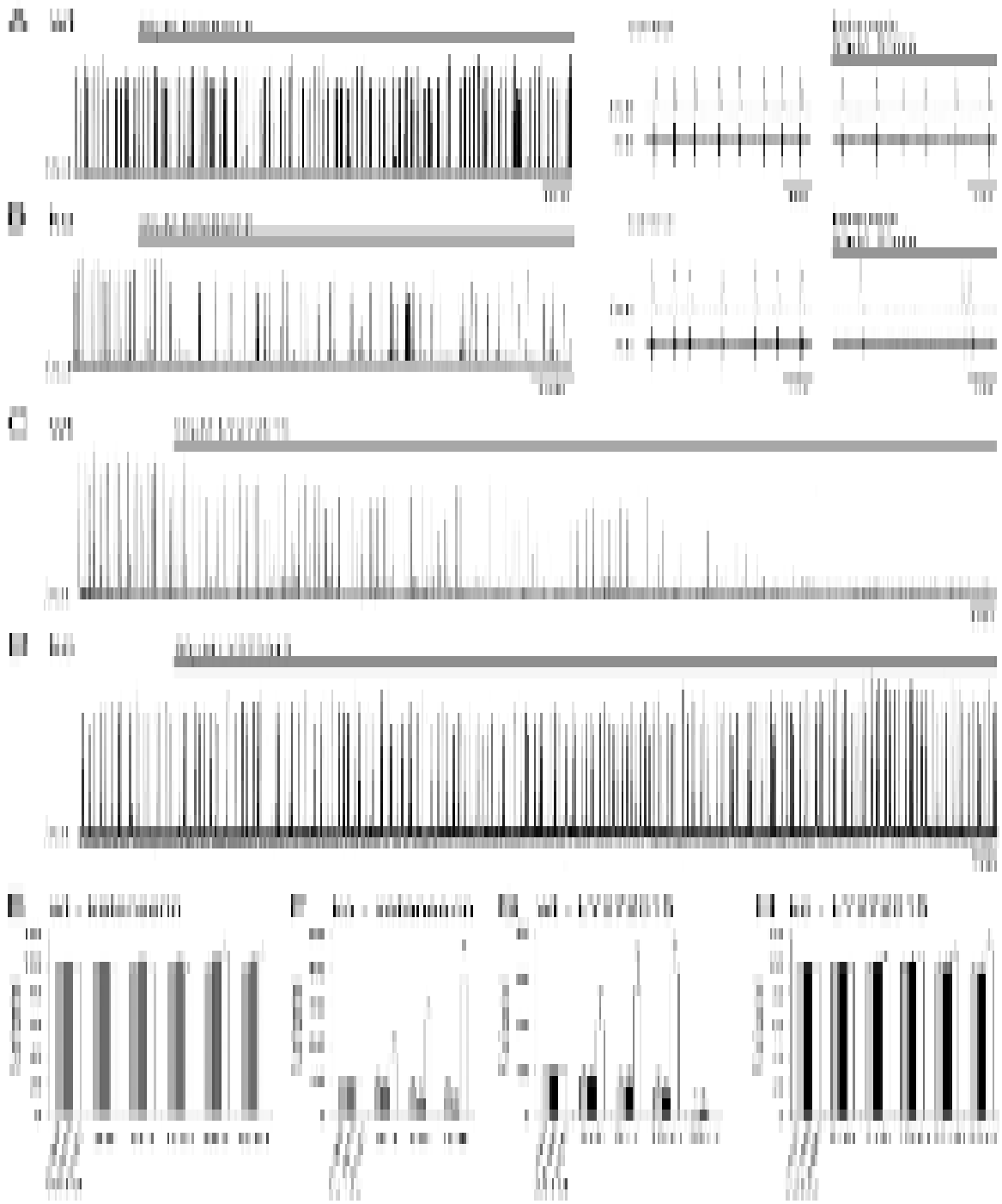

Figure 6
Bath application of the 5-HT_{2A} receptor antagonist ketanserin did not influence regular respiratory activity even after prolonged exposure in wild type mice (wt) preparations (A). In contrast, bath application of ketanserin on 5-HT_{2B} receptor deficient mice (ko) preparations caused an instantaneous irritation of respiratory activity (B). Bath application of the 5-HT_{2B} receptor antagonist LY272015 caused a gradual decrease of amplitude and frequency of respiratory bursts which led to a total stop of respiratory activity in wt preparations (C). In ko preparations, LY272015 exhibited no effects on respiratory activity (D). E - H, Histograms plotting the percentage changes for burst amplitude (grey columns), frequency (black columns), and irregularity (white columns) 2 – 60 min after bath application of the 5-HT_{2A} receptor antagonist ketanserin (E, F) or the 5-HT_{2B} receptor antagonist LY272015 (G, H). * $p > 0.05$ (Student`s paired *t* test) compared with control. Note the differences in scaling.