

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 February 2007 (22.02.2007)

PCT

(10) International Publication Number
WO 2007/019880 A1

(51) International Patent Classification:

A61K 31/55 (2006.01) A61K 31/553 (2006.01)
A61K 31/5375 (2006.01) A61K 31/336 (2006.01)
A61K 31/137 (2006.01) A61P 11/16 (2006.01)
A61K 31/138 (2006.01) A61P 25/00 (2006.01)

(21) International Application Number:

PCT/EP2005/009893

(22) International Filing Date: 18 August 2005 (18.08.2005)

(25) Filing Language:

English

(26) Publication Language:

English

(71) Applicant (for all designated States except US): INSERM
(INSTITUT NATIONAL DE LA SANTE ET DE LA
RECHERCHE MEDICALE) [FR/FR]; 101, rue de Tol-
biac, F-75013 PARIS (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): VILLARD, Lau-
rent [FR/FR]; 10, Tse de la Gaye, F-13009 MARSEILLE
(FR). ROUX, Jean-Christophe [FR/FR]; 1, rue des
Genats, F-13190 ALLAUCH (FR). HILAIRE, Gérard
[FR/FR]; 49, boulevard Baltazar Blanc, F-13015 MAR-
SEILLE (FR).

(74) Agents: VIALLE-PRESLES, Marie José et al.; CABLI-
NET ORES, 36, rue de Saint-Petersbourg, F-75008 PARIS
(FR).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,
MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ,
OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL,
SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC,
VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,
GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: USE OF A SELECTIVE INHIBITOR OF NOREPINEPHRINE REUPTAKE FOR TREATING RESPIRATORY DIS-
ORDERS RESULTING FROM RETT SYNDROME

(57) Abstract: The present invention relates to the treatment or prevention of the breathing disturbances of Rett Syndrome by use
of a selective inhibitor of norepinephrine reuptake.

WO 2007/019880 A1

USE OF A SELECTIVE INHIBITOR OF NOREPINEPHRINE REUPTAKE FOR TREATING RESPIRATORY DISORDERS RESULTING FROM RETT SYNDROME

The present invention relates to the treatment or prevention of the breathing disturbances of Rett Syndrome.

Rett Syndrome is a severe neurological disorder which may account for up to 10% of severe mental retardation of genetic origin in women (Armstrong, 1997). Although a few familial cases have been reported, most of the cases are sporadic with frequent (80-90 %) mutations in the methyl-CpG binding protein2 (*MECP2*) gene (Van den Veyver and Zoghbi, 2000). The disorder typically commences with an apparently normal development until 6 to 18 months of age. Thereafter the patients suffer from a number of neurological symptoms including regression of acquisitions, behavioral disturbances with stereotypic hand movements (Hagberg et al., 1983) and severe breathing irregularities (Eliań and Rudolf, 1991; Kerr, 1992; Woodyatt and Murdoch, 1996; Morton et al., 1997; Cooper et al., 1998; Kerr and Julu, 1999; Julu et al., 2001). Twenty six percent of deaths in Rett girls occur with sudden respiratory arrhythmia (Kerr et al., 1997). The breathing irregularity has puzzled clinicians because of its state-dependency. Breathing is regular during sleep and can switch from highly irregular to regular even during wakefulness (Marcus et al., 1994; Kerr and Witt-Engerstrom, 2001). Because breathing can be regular many clinicians believe that breathing problems are a consequence of disturbed cortical rather than brainstem mechanisms (Marcus et al., 1994) and may thus be behaviourally determined (Eliań and Rudolf, 1991).

Numerous hypotheses have been proposed to explain these respiratory alterations, among which a deficiency in endogenous bioamines (catecholamines and serotonin), but no definitive conclusions have been reached (Nomura et al., 1985; Zoghbi et al., 1985; Riederer et al., 1986; Zoghbi et al., 1989; Lekman et al., 1990; Nielsen et al., 1990; Segawa, 1997; Kerr et al., 1998; Dunn, 2001; Dunn and MacLeod, 2001).

Independently, it has been shown that NE plays a key role in the maturation and modulation of the respiratory network (Viemari et al., 2004), and that 5HT and substance P affect respiratory rhythm (Bou-Flores et al., 2000; Pena and Ramirez, 2002, , 2004). However, no link between these neuromediators and the respiratory disorders observed in Rett Syndrome has been demonstrated.

A mouse experimental model of Rett Syndrome created by genetic invalidation of the *MECP2* gene is available (Guy et al., 2001). The inventors have performed experiments on wild-type and *Mecp2*-deficient mice, in order to test the role of this gene in respiration and in bioaminergic systems. They have observed that adult *Mecp2*-deficient mice show respiratory alterations under the form of fast and slow breathing periods interrupted by apneas of variable duration, and bioaminergic metabolism alterations under the form of a significant reduction of the norepinephrine (noradrenaline) and the serotonin content in the medulla and a drastic reduction of medullary tyrosine-hydroxylase expressing neurons. They

have also performed *in vitro* experiments on the isolated medullary-spinal cord preparations of wild type neonatal mice, and found that that endogenous noradrenaline helps to maintain a normal respiratory rhythm.

On the basis of these findings the inventors have hypothesized that disturbed norepinephrine modulation of the medullary respiratory network may play a major part in the breathing disturbances observed in *Mecp2*-deficient mice and Rett patients, and have searched for treatments allowing to prevented or alleviate said breathing disturbances.

They have found that desipramine, a selective inhibitor of norepinephrine reuptake, efficiently reduced the respiratory alteration occurring in *Mecp2*-deficient mice.

The present invention thus relates to the use of a selective inhibitor of norepinephrine reuptake for preparing a medicament for treating or preventing respiratory disorders resulting from Rett Syndrome.

The present invention also includes a method for treating or preventing respiratory disorders resulting from Rett Syndrome, wherein said method comprises administering to a patient suffering of said disorder an effective amount of a selective inhibitor of norepinephrine reuptake.

A "selective inhibitor of norepinephrine reuptake" is defined herein as a compound having an inhibition constant (K_i , nmol/l) for norepinephrine uptake by the norepinephrine transporter (NET) of less than 100 nM, preferably less than 50 nM, and also having at least a 5-fold, preferably at least a 10-fold, and more preferably at least a 20-fold selectivity for the norepinephrine transporter (NET) over the serotonin transporter (SERT), and over the dopamine transporter (DAT). Selectivity of a compound refers to the ratio of its K_i for serotonin or dopamine uptake by SERT or DAT, respectively, to its K_i for norepinephrine uptake by the norepinephrine transporter (NET). For example, the selectivity of a compound for NET over SERT or over DAT would be expressed as K_i for SERT (or K_i for DAT)/ K_i for NET. The K_i of a compound for a given monoamine transporter can be obtained by methods known in themselves, for instance by competition assays using membranes obtained from cell lines transfected with human gene for the specific monoamine transporter, such as those disclosed by Frazer (1997), Owens et al (1997), or Leonard and Richelson (2000).

Selective inhibitors of norepinephrine reuptake include, by way of non limitative examples, antidepressants such as imipramine, clomipramine, opipramol, prozapine, quinupramine, trimipramine, reboxetine, atomoxetine, amoxepine, caripramine, doxepine and maprotiline. The selective inhibitor of norepinephrine reuptake may be administered orally or parenterally, by any suitable means known to one of ordinary skill in the art. Formulations of suitable for oral administration, may be in the form of discrete units, such as capsules, cachets, or tablets, or in the form of a powder or granules for reconstitution. Formulations suitable for oral or parenteral administration may be in the form of a solution or

a suspension or an emulsion in an aqueous liquid or nonaqueous liquid. All these formulations may be prepared by any of the methods known in the art of pharmacology.

The doses of selective inhibitor of norepinephrine reuptake effective for alleviating respiratory disorders resulting from Rett Syndrome are generally of the same order as the doses used in the treatment of depression.

For instance, in the case of oral administration of desipramine, doses of 10 to 30 mg a day are generally suitable for children, and 100 to 200 mg a day are generally suitable for adults. This quantity may be administered once a day, or if desired it may be divided into two or more administrations.

The appropriate daily dosage, route of administration, and administration regimen can easily be determined for each patient by the physician, taking in account all the particulars of the patient, such as the age, the weight, and general medical condition of the patient, and the severity of the respiratory disorders.

In addition to containing the selective inhibitor of norepinephrine reuptake and the standard and well known pharmaceutical carriers and/or excipients, all of the above formulations may contain other therapeutically-active substances, for instance antagonists of presynaptic α_2 adrenergic autoreceptors, such as Mirtazapine.

The present invention is further illustrated by the examples that follow, without being limited, however, to the specific details of these examples.

FIGURE LEGENDS

Figure 1:

A-D: The traces show typical plethysmographic recordings of breathing (inspiration upward) performed in the same unanesthetized, quiet *Mecp2*^{-/-} adult mice at different ages, i.e. at 30 days (A), 45 days (B), 55 days (C) and 59 days (D). This animal died when 60 days old; (d-30), (d-15), (d-5) and (d-1) refer to the number of days before death. E: Sequential plot of cycle period values (TTOT in s) of 80 consecutive respiratory cycles recorded in the unanesthetized *Mecp2*^{-/-} mouse shown in A-D at 30 days (lozenges), 5 days (open squares) and 1 day (triangles) before death.

Figure 2:

A-D: Plethysmographic recordings of breathing (inspiration upward) at about 6 weeks of age in unanesthetized, quiet wt (A) and *Mecp2*^{-/-} adult mice (B-D). *Mecp2*^{-/-} mice show a mixture of slow and fast respiratory rhythm (B) and periods of short-lasting (C) and long-lasting apneas (D). E-F: Sequential plot of TTOT values (in s) of 80 consecutive respiratory cycles recorded in wt (E) and *Mecp2*^{-/-} (F) adult mice reveal that TTOT values are regular in the wt mouse but scattered in the *Mecp2*^{-/-} mouse.

Figure 3:

Distribution of T_{TOT} and V_T/B values recorded in unanesthetized paired wt and *Mecp2*^{-/-} mice from the same litter (recording performed the same day). Frequency histograms represent the number of occurrences (ordinate) of T_{TOT} (A1, A2, abscissa) and V_T/B (B1, B2, abscissa) values on 100 consecutive respiratory cycles during quiet breathing in the wt mouse (A1, B1) and the *Mecp2*^{-/-} mouse (A2, B2) from one pair of litter mates.

Figure 4 :

A: Plethysmographic recordings obtained from the same *Mecp2*^{-/-} mouse when unanesthetized (A1) and anesthetized (A2). **B-C:** Frequency histograms present the number of occurrences of T_{TOT} (B1, B2) and V_T/B (C1, C2) values on 100 consecutive respiratory cycles during awake (B1, C1) and anesthetized (B2, C2) conditions in the same *Mecp2*^{-/-} mouse.

Figure 5:

A: Schematic of a transverse brainstem slice that spontaneously generates respiratory rhythmic activity. Upper trace: Extracellular population activity recorded from the VRG. Lower trace: Integrated activity obtained from the extracellular population activity.

Abbreviations: X: vagus nuclei, XII: hypoglossal nuclei, PBC: Pre-Bötzinger complex area, SP5: spinal trigeminal nucleus, VRG: ventral respiratory group, IO: inferior olive

B1: Integrated regular respiratory activity from a wt mouse. **B2:** *Mecp2*^{-/-} mouse with an irregular respiratory rhythm. **C-D:** Sequential plot (C) and frequency histograms (D) of cycle period (s) obtained for 80 consecutive VRG bursts recorded in brainstem slices from wt (C1, D1) and *Mecp2*^{-/-} (C2, D2) mice. Note cycle period variability in the *Mecp2*^{-/-} mouse.

Figure 6:

A1: Integrated population activity from a wt slice preparation before application of NE.

A2: In the same animal, exogenous application of NE [20 μ M] increases the respiratory rhythmic frequency. **B1:** Integrated population activity from a *Mecp2*^{-/-} mouse before application of NE. **B2:** Exogenously applied NE [20 μ M] significantly increases the regularity and frequency of respiratory rhythmic activity.

C: Examples of sequential cycle period histograms for a wt (C1), a *Mecp2*^{-/-} mouse before NE application (C2) and the same *Mecp2*^{-/-} mouse in the presence of NE (C3). Each histogram represents cycle period values (ordinate, sec) calculated for 80 consecutive cycles (abscissa). Note that the cycles period values become more regular after adding NE.

Figure 7:

A-D: Schematic representation of a medullary slice of adult wt mice and enlargements showing neurons of the X and XII motor nuclei that express the synthesis enzyme choline acetyltransferase (A), 5HT neurons (C) and neurons of the dorsal A2/C2 (B-B') and ventral A1/C1 (D) groups that express Tyrosine Hydroxylase (TH). *Mecp2*^{-/-} mice present a significant decrease of TH neurons in A1/C1 (not shown) and A2/C2 cell group (B-B').

Abbreviations: Amb, ambiguus nucleus; Ap, area postrema; Cu: cuneate nucleus; LRt, lateral reticular nucleus; Py, pyramidal tract; SolM; nucleus solitary tract; Sp5: spinal trigeminal nucleus; X, dorsal vagal motor nucleus; XII, hypoglossal motor nucleus.

Figure 8:

Number of apneas/hour in *Mecp2* deficient mice receiving i.p. injections of physiological serum (placebo, grey square) or desipramine hydrochloride (black diamond), before treatment (Av), on the first day of treatment (J) and at different times of treatment(#j).

MATERIALS AND METHODS**Animals breeding and genotyping.**

Experiments were performed on mice using the mouse model (strain B6.129P2(C)-*Mecp2*^{tm1-1Bird}) for Rett Syndrome developed by A. Bird (Guy et al., 2001). The mice were obtained from the Jackson Laboratory and maintained on a C57BL/6 background. Hemizygous mutant males were generated by crossing heterozygous knockout females to C57BL/6 males. All our experiments were performed in hemizygous *Mecp2* deficient male. Although Rett syndrome in humans affects female patients, most researchers use *Mecp2*^{-/-} male mice for their studies. This choice is dictated by the fact that the *Mecp2* gene is X-linked in mouse and in humans, and females will thus have a different amount of normally *Mecp2*-expressing cells depending on their X-chromosome inactivation profile. Since the proportion of *Mecp2*-deleted X chromosomes that will be inactivated in a given female animal is unpredictable, we decided to use *Mecp2*^{-/-} male mice that correspond to a complete absence of the *Mecp2* gene product in all cells (i.e. a real null phenotype). Genotyping was performed by routine PCR technique according to Jackson Laboratory protocols. Other experiments were performed on wt C57BL/6 strains (Iffa-Credo breeding centre, Saint Germain-sur-l'Arbresle, France). The experimental procedures were carried out in keeping with the European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC) and with the Institutional Animal Care and Use Committee at The University of Chicago. Unless stated, all the chemical compounds were from Sigma (either St. Louis, MO, USA or St Quentin, France).

Plethysmographic recording of mouse breathing pattern

As reported elsewhere in detail (Burnet et al., 2001; Viemari et al., 2004), the breathing pattern was recorded from unrestrained mice by whole-body barometric plethysmography. The animal and reference chambers (200 ml and 25 ml for adult and young mice, respectively) were immersed in a temperature-regulated water bath, and maintained at 26 – 28 °C (temperature sensor Checktemp 1, Hanna Instruments, Lingolsheim, France). The spirogram was obtained by recording the pressure difference between the two chambers (Validyne CD 15, frequency response: DC to 1000 Hz, Northridge, CA, USA). The signal was amplified, filtered (DC-50 Hz), fed to an analog-to-digital converter (sampling frequency 1 kHz), and stored on a PC disk via Spike 2 interface and software (Cambridge Electronic Design, Ltd, Cambridge, UK). For each mouse, successive 3-minute plethysmographic measurements were performed until the animal was quiet (i.e. without limb, body and head movements). Only the recording periods during which the animals were quiet were analyzed. The total respiratory cycle duration (TTOT) and the tidal volume (VT) were measured for each cycle of a recording of a 100 consecutive respiratory cycles. In the figures, VT values were normalized and expressed as the ratio of the VT divided by the body weight (VT/B). The measurements were interrupted every 3 minutes to flush the animal chamber with air for 1 minute. Controls performed in some experiments showed that oxygen and carbon dioxide fractions were normal in the animal chamber (Portable Gaz Analyser KG850, Hitech Instruments, Luton, England). During on-going experiments, breathing was recorded in a whole-body flow plethysmograph (EMKA Technologies, Paris, France) in which a constant flow pump connected to the animal chamber ensures proper and continuous inflow of fresh air, avoiding interruption of recording to flush air in the animal chamber. Similar results were obtained in *Mecp2* deficient mice with both types of plethysmograph. To test the effect of anesthesia on breathing of *Mecp2*^{-/-} mice, some plethysmographic recordings were performed in slightly anesthetized *Mecp2*^{-/-} mice which received half surgical doses of sodium pentobarbitone (30 mg kg⁻¹ i.p., Sanofi, France).

In vitro electrophysiological study of medullary respiratory network in young mice

Experiments were performed on wt and *Mecp2*^{-/-} mice at postnatal days fourteen to twenty one (P14-P21) using a slice preparation technique previously described in details (Pena and Ramirez, 2002). Throughout the experiments the experimenter was blind to the mouse genotype. Briefly, the animals were decapitated under ether anesthesia and the isolated brainstem placed in ice-cold artificial cerebral-spinal fluid (ACSF) bubbled with carbogen (95% O₂ and 5% CO₂). The ACSF contained (in mM): 128 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose, pH of 7.4. The brainstem was glued rostral end-up onto an agar block, was mounted into a vibratome (Leica Microsystems, Waukegan, IL) and serially sliced until disappearance of the facial nucleus, and appearance of the inferior olive, the nucleus ambiguus and the hypoglossal nucleus. A

single 650 μm thick slice was then taken and used for study. We refer to the area encompassed in the slice as the ventral respiratory group (VRG). Slices were transferred into a recording chamber, continuously superfused with oxygenated ACSF and maintained at a temperature of $29 \pm 0.5^\circ\text{C}$. To initiate and maintain fictive respiratory rhythmic activity the potassium concentration of the perfusate was raised from 3 to 8 mM over 30 minutes (Tryba et al., 2003). The population activity from the VRG neurons was recorded with suction electrodes positioned on the surface of the slice and was used as a marker for inspiratory activity. The signals were amplified, filtered (low pass 1.5 KHz, high pass 250 Hz), rectified and integrated (time constant of 60 ms). All recordings were stored on a computer using AxoTape (Version 2.0, Axon Instruments, Union City, CA) and analyzed offline using customized analysis software written with IGOR Pro (Wavemetrics, Lake Oswego, Oregon).

In some experiments, norepinephrine was added to carbogenated ACSF. The normal ACSF was changed to ACSF containing NE for 5–10 min and the resulting alterations in frequency and stability of VRG bursts were analyzed as reported below.

Biochemical analysis

Twelve *Mecp2*^{-/-} mice and eighteen wt mice were killed with a lethal pentobarbitone injection (300 mg kg^{-1} i.p.), and their brains were dissected out within 5 minutes of their last breaths. The forebrain, pons and medulla were separated, weighted, and kept at -80°C until biochemical analysis. Each sample was homogenized in cold trichloroacetic acid (5% in H_2O ; 200 μl for pons and medulla and 1000 μl for forebrain) with a micropotter. The cellular suspension was then centrifuged (10 minutes, 600 g, 5°C), the supernatant was collected and diluted by adding a volume of an antioxidant solution (0.65 mM of ascorbic acid and 0.35 mM of EDTA in H_2O) corresponding to one-fifth of the supernatant volume. High Performance Liquid Chromatography (UVK Lab., Paris, France) coupled with electrochemical detection was used to measure the endogenous concentrations of norepinephrine, dopamine and serotonin. The carbon electrode was at a potential of +650 mV against the Ag/AgCl reference electrode of the electrochemical detector (Model 105, Precision Instruments, France) and the sensitivity of the detection was set up to 0.05 nA V^{-1} . The compound concentrations were also measured in 1 μl standard samples injected by an autosampler Biotek 565 (UVK Lab) into an hypersil ODS Column (200 x 3 mm; 3 μm) (Phymep, Paris, France) in which the polar mobile phase (in mM: citric acid 120, potassium hydrogenophosphate 430, heptane sulfonic acid 4.2, EDTA 1.7, and 10 % methanol in H_2O) was delivered at a rate of 0.2 ml min^{-1} . The endogenous concentrations were expressed in nM/mg of brain sample.

Immunohistofluorescence

One and two months old mice were anesthetized with a lethal pentobarbitone injection (300 mg kg^{-1} i.p.) and transcardially perfused (chilled saline for 1 min followed by PBS 0.1 M containing 4% paraformaldehyde for 10 min). Brains were

postfixed for 5 h and placed overnight in PBS containing 20% sucrose. For neonatal mice, brains were dissected and fixed by immersion during 12 h and placed overnight in PBS containing 20% sucrose. Medullary coronal sections were cut on cryostat (20 μ m) and one every successive five slices was arranged serially on a slide. Sections were permeabilized (0.15% TritonX⁻¹⁰⁰), blocked with 7% normal goat serum (NGS), and incubated overnight at 4°C with primary antibody in PBS containing 3.5% serum, 0.15% Triton X⁻¹⁰⁰. Sections were washed, incubated with secondary antibody in PBS containing 3.5% serum, 0.15% Triton X⁻¹⁰⁰, and re-washed. The sections were subsequently mounted in Prolong antifade (Molecular Probes, Eugene, Oregon). Tyrosine hydroxylase (TH), serotonin (5HT) and choline acetyl transferase (ChAT) were probed with affinity-purified rabbit polyclonal antibodies (1:1000, Institut J. Boy, Reims, France; 1:1000, Sigma-Aldrich and 1:500, Chemicon, respectively). Goat-anti-rabbit Alexa 488 (1:200) from Molecular Probes, Eugene, Oregon) was used as secondary antibodies. Each TH, 5HT and ChAT antibody was applied to only one every five successive sections. The nuclei of immunolabelled cell bodies were counted with an Olympus BX50 microscope equipped with a high-resolution digital camera (excitation 488 nm; detection 515-540 nm band-pass filter). The number of TH-positive neurons in the ventral A1/C1 and dorsal A2/C2 groups, 5HT-positive neurons in the median B1-B2 and the lateral B3 serotonergic groups and ChAT-positive neurons in the X and XII motor nuclei, was determined in every immunolabelled sections. For neonatal and one month old mice, only the TH analysis was performed. The number of TH-neurons is expressed as the mean $\pm$ sem.

Desipramine treatment

Mecp2^{-y} mice having 80-100 apneas lasting more than 1 second per hour, or wt C57BL/6 mice of the same age (i.e. about 45 days) received daily (between 9.30 and 10.30 a.m.) an intraperitoneal injection of desipramine hydrochloride (10 mg kg⁻¹; Sigma-Aldrich) diluted in physiological serum. Control mice received a daily injection of physiological serum.

Statistical Analysis

The data were analyzed with SPSS software (SPSS Science Software GmbH, Erkrath, Germany). For all tests, the statistical significance was taken at $p \leq 0.05$.

Variability of respiratory cycle period.

To analyze the variability of the respiratory cycle period, we used several statistical tests depending on experimental conditions. For *in vivo* data, we used the one-tailed Moses rank-like test for scale differences (Siegel and Catellan, 1989) to compare the TTOT or VT distribution (100 respiratory cycles) between paired animals (one *Mecp2*^{-y} mouse and in its wt littermate recorded on the same day) or between paired conditions (un anesthetized and anesthetized *Mecp2*^{-y} mouse). Then, the p values obtained for each pair were combined by the Edgington procedure as described by (Krauth, 1990). Briefly, if k independent

comparisons give k p-values, respectively $p_1, p_2 \dots p_k$, the p-value for the combined test, p_T , is calculated according to the equation $p_T = s^k / k!$ in which $s = p_1 + p_2 + \dots + p_k$. The p_T value gives the probability that the variability was higher in one than the other condition. For *in vitro* data, we calculated both the coefficient of variation (Cvd) and the irregularity score (IS) of cycle period of VRG bursts produced in slice preparations. The Cvd was defined as the ratio between the SD and the mean cycle period measured during 80 successive respiratory cycles (Viemari et al., 2004); mean Cvd values are given in the text but not statistically compared because we lack adequate tests. The IS was defined for each cycle by applying the formula for consecutive cycle period values $100 \cdot \text{ABS}(P_n - P_{n-1}) / P_n$, with P being the period of the n th respiratory cycle (Telgkamp et al., 2002); mean IS values for wt and *Mecp2*^{-/-} mice were compared by Student's T-test. In addition, we also used the one-tailed Moses rank-like test for scale differences followed by the Edgington procedure to compare the distribution of respiratory cycle period between paired wt-*Mecp2*^{-/-} slices (mice from same litter).

Other data.

For biochemical and histological data, results are given as medians $\pm$ quartile deviation (i.e., half of the difference between the 75th and the 25th percentile) and the statistical differences between the wt and *Mecp2*^{-/-} mice were analyzed by the non-parametric Mann-Whitney U test. For pharmacological data, the frequency changes induced by ACSF containing NE were analyzed by one-way ANOVA (experimental conditions: control and NE application) for repeated measures in the same subjects, followed by Tukey's tests as multiple-comparisons procedure. The effect of NE application on the IS was analyzed by a two-way ANOVA, [the factors are strain (wt or *Mecp2*^{-/-} mice) and experimental conditions (control, NE application and recovery)] for repeated measurements in the same subjects with only one repeated factor (experimental conditions) followed by Tukey's tests as multiple comparisons procedure.

EXAMPLE 1: EFFECT OF MECP2 DEFICIENCY ON THE BREATHING PATTERN IN ADULT MECP2^{-/-} MICE

Plethysmography recordings were performed in 25 unrestrained *Mecp2*^{-/-} mice, 15 young mice that were recorded at least once before one month of age and thereafter sacrificed for other analysis and 10 adult mice that were recorded several times between one and two months of age.

None of the 15 young *Mecp2*^{-/-} mice presented severe breathing disturbances when studied at postnatal days 4-5 (P4-P5, $n=2$), P10-P14 ($n=8$) and P21 ($n=5$). Most of them had normal breathing pattern with stable cycle period although short apneas lasting 1-2 s were occasionally observed in one of the eight P10-P14 mice and in three of the five P21 mice (median 3 apneas during 15 min recording sessions; range 2-5), intermingled with respiratory cycles of variable period.

At four weeks of age, the 10 adult *Mecp2*^{-/-} mice also had breathing patterns that were not obviously different from that of their wt littermate (Fig.1A and 2A). However in the following week, they began to develop breathing disturbances that worsened until death (Fig.1B-1D). Appearance and progression of these breathing disturbances were highly variable from individual to individual. As already reported (Guy et al., 2001), *Mecp2*^{-/-} mice fail to thrive and their lifespan is short (54 days) although some animals survive until three months of age. In our sample, adult *Mecp2*^{-/-} mice had a significantly reduced body weight (23 ± 1 g and 15 ± 2 g for wt and *Mecp2*^{-/-} mice) and a short lifespan. Eight *Mecp2*^{-/-} mice died before 2 months of age (median 54 days, range 32-60 days), two survived until 67 and 89 days and all presented breathing disturbances that developed during the studied period. At 6 weeks of age, i.e. about 15 days before death, the mean breathing frequency was not significantly different in wt and *Mecp2*^{-/-} mice (3.25 ± 0.28 Hz vs. 2.95 ± 0.50 Hz, respectively) but *Mecp2*^{-/-} mice displayed alternating periods of fast and slow respiratory frequencies (Fig. 2B) and apneas of variable duration (Fig. 1B; Fig. 2C, 2D). (median 6 apneas lasting more than 1 second during 15 min recording sessions; range 3-25). Indeed, the respiratory cycle period was more stable in wt than *Mecp2*^{-/-} mice (Fig. 2E, 2F). In *Mecp2*^{-/-} mice, the apneas were sometimes preceded by an increase in the breathing frequency or by a large inspiration (Fig.2D). However, this was not always the case (Fig. 1B) and the occurrence of apneas was therefore unpredictable. In addition, a given *Mecp2*^{-/-} mice that displayed breathing disturbances during 2-3 consecutive recording sessions of 15 min might transiently show an apparently normal breathing during the next recording session. One week later, i.e. about 7 days before death, breathing disturbances were almost permanent and very severe (Fig. 1C, 1D), with a significantly reduced mean breathing frequency (3.33 ± 0.23 Hz vs. 1.55 ± 0.38 Hz, for wt and *Mecp2*^{-/-} mice, respectively) and very frequent long lasting apneas (median 10 apneas lasting more than 1 second during 15 min recording sessions; range 5-75).

The variability of the lifespan, as well as the onset and progression of breathing disturbances in *Mecp2*^{-/-} mice impaired a statistical study on the whole sample of 10 mutants. We focused our statistical analysis on five pairs of *Mecp2*^{-/-} and wild-type (wt) littermates whose breathing patterns were recorded on the same day at least once every week from one month of age up to the spontaneous death of the *Mecp2*^{-/-} mouse. We compared the stability of tidal volume V_T (divided by the body weight, V_T/B) and respiratory cycle period (T_{TOT}) in *Mecp2*^{-/-} and wt littermates. Although T_{TOT} varied from individual to individual, it was very regular in a given wt mouse with only minor variations (Fig. 2A, 2E) but very irregular in a given *Mecp2*^{-/-} mouse (Fig. 2B, 2F). As illustrated in the frequency histograms (Fig. 3), the distribution of the V_T/B and T_{TOT} values was less dispersed in wt than in *Mecp2*^{-/-} mice. At about 15 days before the *Mecp2*^{-/-} death, the statistical analysis of the raw values with the Moses rank-like test for scale differences revealed a significant difference

between the members of each pair of T_{TOT} and V_T/B values. The Edgington procedure, which allows generalization of the statistical analysis to the tested populations, confirmed a significant difference between *Mecp2*^{-/-} and wt mouse populations.

Thus, *Mecp2* deficiency significantly alters the breathing pattern in adult mice, inducing a highly variable cycle period. *Mecp2*^{-/-} mice were however capable of generating regular breathing in the presence of light anesthesia. This effect was analyzed in 5 *Mecp2*^{-/-} adult mice that displayed breathing disturbances (Fig. 4A1). Ten minutes following anesthesia onset, breathing activity became regular and stable with no apneas (Fig. 4A2). The distributions of T_{TOT} and V_T were significantly different in unanesthetized (Fig. 4B1 and 4C1) and anesthetized conditions (Fig. 4B2 and 4C2). The significance was confirmed with the Edgington procedure.

EXAMPLE 2: EFFECT OF MECP2 DEFICIENCY ON *IN VITRO* RESPIRATORY RHYTHMOGENESIS IN BRAINSTEM SLICES FROM YOUNG MECP2^{-/-} MICE

In young mice, the numerous peripheral inputs that impinge onto the medullary respiratory network and regulate its activity *in vivo* may have masked or compensated some central respiratory deficits.

Therefore, we isolated a critical portion of the respiratory rhythm generating network from these peripheral inputs by using a transverse brainstem slices preparation. This slice contains neurons of the ventral respiratory group (VRG, Fig. 5A) that are thought to form a network that generates the basic respiratory rhythm. The spontaneous generated bursts of population activity of this region correspond to fictive inspiratory activity.

The *in vitro* generation of rhythmic VRG bursts was compared between slices from 14 wt and 9 *Mecp2*^{-/-} mice aged from 14 to 21 postnatal days (P14-P21), i.e. at a time when plethysmographic recordings did not yet reveal drastic breathing alterations *in vivo*. Neither the mean duration of VRG bursts (0.42 ± 0.02 s and 0.43 ± 0.03 s for wt and mutants) nor their mean frequency (0.22 ± 0.02 Hz and 0.29 ± 0.05 Hz for wt and mutants) were statistically different. However, the cycle period of VRG bursts was highly irregular in *Mecp2*^{-/-} slices when compared to wt slices (Fig. 5B1-B2) as illustrated by sequential cycle period scatter plots (Fig. 5C1-C2). The irregularity of the cycle period was quantitatively assessed by calculating both the coefficient of variation of cycle period CVd ((Viemari et al., 2004) and the irregularity score IS (Telgkamp et al., 2002). Both values were about two- fold higher in *Mecp2*^{-/-} (0.56 ± 0.07 and 54 ± 6 for CVd and IS, respectively) than in wt slices (0.27 ± 0.03 and 28 ± 2 for CVd and IS, respectively). In addition in five paired slices from wt and *Mecp2*^{-/-} littermates, Moses rank-like test for scale differences confirmed that the distribution of the VRG cycle period was less dispersed in wt (Fig. 5D1) than in *Mecp2*^{-/-} slices (Fig. 5D2) and the Edgington procedure revealed a significant difference between the two populations.

EXAMPLE 3: EFFECT OF EXOGENOUSLY APPLIED NOREPINEPHRINE ON THE RESPIRATORY RHYTHM IN BRAINSTEM SLICES OF MECP2-/Y OR WILD TYPE MICE

Because norepinephrine (NE) is known to play a role in respiratory rhythm regulation (Viemari et al., 2004), we examined whether the irregularity of the cycle period in slices from *Mecp2*^{-/-} mice may result from a disturbance of NE mechanisms. Exogenously applied NE significantly increased the VRG bursts in both wt and *Mecp2*^{-/-} slices by 79 ± 17 % (n=5, Fig 6A) and 123 ± 53 % (n=5, Fig 6B), respectively. In the presence of NE, the mean VRG bursts did not differ in wt and *Mecp2*^{-/-} slices (0.39 ± 0.01 Hz and 0.49 ± 0.11 Hz respectively). Moreover, NE application eliminated the rhythm irregularity in all examined slices from *Mecp2*^{-/-} mice (Fig 6B2, 6C2-C3), halving the CVd and IS values (0.29 ± 0.1 and 31 ± 1 , n=5, respectively). By contrast, NE application in slices from wt mice had no effects on CVd and IS values. Thus in the presence of NE, slices from wt and *Mecp2*^{-/-} mice produced rhythmic VRG bursts with a statistically similar frequency and regularity. Furthermore, the cycle period of VRG bursts remained regular in mutant mice for at least 5¹⁰ minutes after washout of NE (0.33 ± 0.04 and 33 ± 3 , n=3 for CVd and IS respectively), while the VRG rhythm returned to control values (0.18 ± 0.03 Hz, n=3).

Thus, these *in vitro* results in slices from wt and *Mecp2*^{-/-} mice reveal that *Mecp2* deficiency alters the cycle period stability and that application of exogenous NE restores the cycle period stability.

EXAMPLE 4 : EFFECT OF MECP2 DEFICIENCY ON ENDOGENOUS BIOAMINES IN MECP2-/Y MICE

We examined whether *Mecp2*^{-/-} mice have a deficiency in NE systems that could contribute to their breathing disturbances.

First, we assessed the endogenous concentrations of NE, dopamine and serotonin (5HT) using HPLC analysis of the medulla, pons and forebrain of 2 months old wt (n=12) and *Mecp2*^{-/-} mice (n=7), which had severe respiratory problems similar to those illustrated in Fig. 1C-D. The results are shown in Table I.

TABLE I

		Wt	P	Mecp2 ^{-/-}
2 months				
Content	NE	13.5 ± 2.3 (n=12)	*	7.0 ± 4.3 (n=7)
	5HT	10.1 ± 1.6 (n=12)	*	6.4 ± 2.6 (n=7)
	DA	0.6 ± 0.2 (n=12)	ns	0.5 ± 0.2 (n=7)
1 month				
Content	NE	15.0 ± 1.6 (n=6)	*	9.2 ± 0.8 (n=5)
	5HT	10.9 ± 1.8 (n=6)	*	8.1 ± 1.5 (n=5)
	DA	0.8 ± 0.2 (n=6)	ns	0.6 ± 0.3 (n=5)

The results are expressed as medians $\pm$ quartile deviation in nM/mg; asterisks and ns indicate significant and non-significant differences, respectively, between wt and *Mecp2*^{-/-} mice of the same age.

In the medulla, NE and 5HT concentrations were significantly lower in *Mecp2*^{-/-} than in wt mice (48 % and 37 %, respectively). By contrast, the dopamine concentration was not different (Table I). In pons and forebrain, NE, dopamine and 5HT concentrations did not differ in wt and *Mecp2*^{-/-} mice (data not shown).

Second, we immuno-labeled medullary neurons that express the catecholamine synthesizing enzyme, tyrosine hydroxylase (TH), in five paired wt and *Mecp2*^{-/-} littermates. TH-neurons were found in the ventral A1/C1 (Fig. 7D) and dorsal A2/C2 (Fig. 7B-B') catecholaminergic groups of wt and *Mecp2*^{-/-} mice. Neuron counting revealed a significantly reduced number of TH-neurons in *Mecp2*^{-/-} mice. The number of TH-neurons was significantly reduced in the A1/C1 group of *Mecp2*^{-/-} mice (302 ± 70 and 211 ± 48 neurons for wt and *Mecp2*^{-/-} mice, respectively). In the entire A2/C2 group (from 2 mm caudal to 2 mm rostral to the obex), the number of TH-neurons was also significantly reduced in *Mecp2*^{-/-} mice (325 ± 19 and 204 ± 9 TH-neurons for wt and *Mecp2*^{-/-} mice, respectively). The number of immuno-labeled neurons expressing 5HT in the median B1-B2 groups and the lateral B3 group was not significantly different between wt and *Mecp2*^{-/-} mice (244 ± 26 and 228 ± 26 , respectively; Fig. 7C). The number of immuno-labeled neurons expressing ChAT in the X and XII motor nuclei (Fig. 7A) was not significantly different between wt and *Mecp2*^{-/-} mice (235 ± 62 and 213 ± 90 in the X motor nucleus and 280 ± 63 and 295 ± 79 in the XII motor nucleus, for wt and *Mecp2*^{-/-} mice, respectively).

Third, we assessed the endogenous concentrations of endogenous bioamines by HPLC analysis of eight wt and seven *Mecp2*^{-/-} mice at one month of age. At this early age, these *Mecp2*^{-/-} mice presented no (n=2) or only minor alterations (n=5) of the respiratory rhythm stability and few apneas (3-6 apneas lasting more than 1 second during 15 min recording sessions). However, their NE concentrations were already significantly lower in mutants than of wt mice (40 %). This reduction was observed in all five studied *Mecp2*^{-/-} mice, i.e. three with slight breathing alterations and two with unaltered breathing. By contrast, 5HT and dopamine medullary concentrations were normal in all examined mice (Table I). At one month of age, neuron counting in two paired wt and *Mecp2*^{-/-} young littermates revealed that the number of TH-neurons in the dorsal A2/C2 area of the *Mecp2*^{-/-} mice was about 60 % of the wt values (345 vs. 185 and 393 vs. 269 neurons for the two pairs of wt and *Mecp2*^{-/-} mice, respectively). In the ventral A1/C1 area, the *Mecp2*^{-/-} count was weak in the mutant from one pair (236 vs. 144) but in the normal range in the mutant from the other pair (280 vs. 260). Finally we counted the number of TH-neurons in the medulla of three paired wt and *Mecp2*^{-/-} neonates (three days old) that displayed a normal breathing. Although a larger number of TH-neurons was found in neonates than in adults, no significant differences were

observed between wt and *Mecp2*^{-/-} neonates (A1/C1 neurons: 414 ± 63 vs. 382 ± 13 neurons for wt and *Mecp2*^{-/-} neonates; A2/C2 neurons: 485 ± 61 vs. 458 ± 27 neurons for wt and *Mecp2*^{-/-} neonates).

Thus, *Mecp2* deficiency induces a specific reduction of the medullary NE contents in adult mice with a reduction of number of medullary NE neurons that occurs postnatally during the first month of age and precedes the occurrence of drastic breathing alterations.

EXAMPLE 5 : EFFECT OF DESIPRAMINE ON THE ALTERATIONS OF THE BREATHING PATTERN INDUCED *MECP2* DEFICIENCY.

Mecp2^{-/-} mice were separated in 2 groups, and the breathing pattern of the mice of both groups was recorded by barometric plethysmography.

The results are shown in Table II below:

TABLE II

Days after birth	Number of apneas /hour	
	Group I	Group II
30 days	40,8	38,5
45 days	85,9	110,2

When the number of apneas/hour reached 80-100, desipramine hydrochloride (10 mg kg^{-1}) or physiological serum (placebo) were administered daily by i.p injection respectively to *Mecp2*^{-/-} mice of Group II and of Group I.

The breathing pattern of the mice of both groups was recorded by barometric plethysmography, up to 80 days after the beginning of the treatment.

The results are illustrated by Table III below, and Figure 8.

TABLE III

Days of treatment	Number of apneas /hour	
	Group I (Placebo)	Group II (desipramine)
D (45 days after birth)	85,9	110,2
+3d	344,1	76,9 *
+5d	366,4	60,6 *
+10d	323,3	74,2 *
+15d	412,7	80,6 *
+20d		51,1
+30d		67,3
+35d		62,4
+40d		148,2
+80d		351,1

These results show that the number of apneas per hour increases significantly between 30 days and 45 days after birth (beginning of the treatment). After the beginning of the treatment, the number of apneas keeps on increasing in control mice, up to a very high level. In treated mice, the number of apneas remains stable and even decreases for a long time.

The survival of the mice in both groups was also recorded. The results are shown in table IV below.

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TABLE IV

	Mecp2 mice+Placebo	Mecp2 mice+desipramine
Survival	61,25 ± 5,7	90.6± 11,2 *

The mean survival of mice treated with desipramine is 48% higher than the mean survival of control mice (* P>0,05).

In the case of wt C57BL/6 treated in the same way, no effect of the desipramine treatment on the number of apneas was observed.

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CLAIMS

1) Use of a selective inhibitor of norepinephrine reuptake for preparing a medicament for treating or preventing respiratory disorders resulting from Rett Syndrom.

2) The use of claim 1, wherein said selective inhibitor of norepinephrine reuptake is selected among imipramine, clomipramine, opipramol, prozapine, quinupramine, trimipramine, reboxetine, atomoxetine, amoxepine, carpipramine, doxepine and maprotiline..

3) The use of any of claims 1 or 2 wherein said selective inhibitor of norepinephrine reuptake is associated with an antagonist of presynaptic autoreceptors.

4) The use of claim 3, wherein said antagonist of presynaptic autoreceptors is Mirtazapine.

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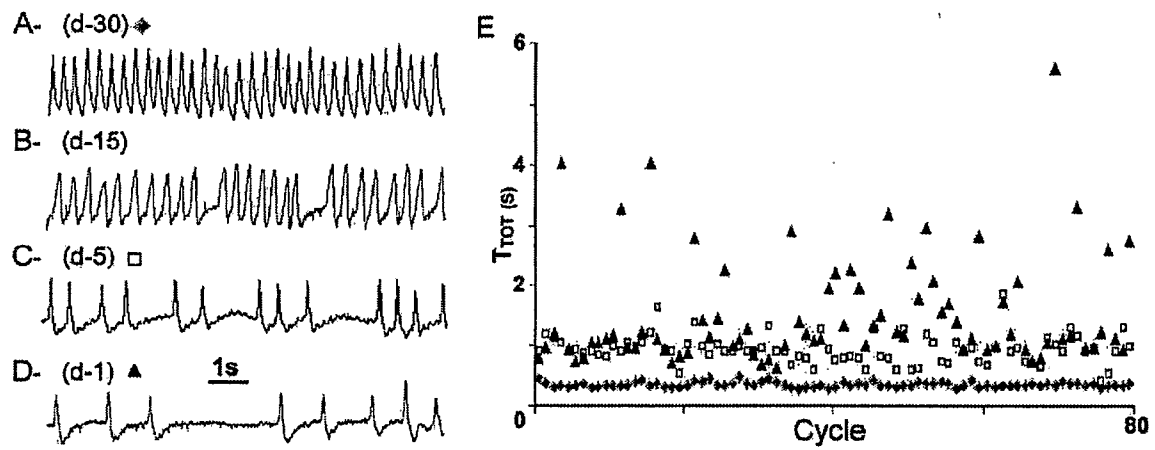


Figure 1

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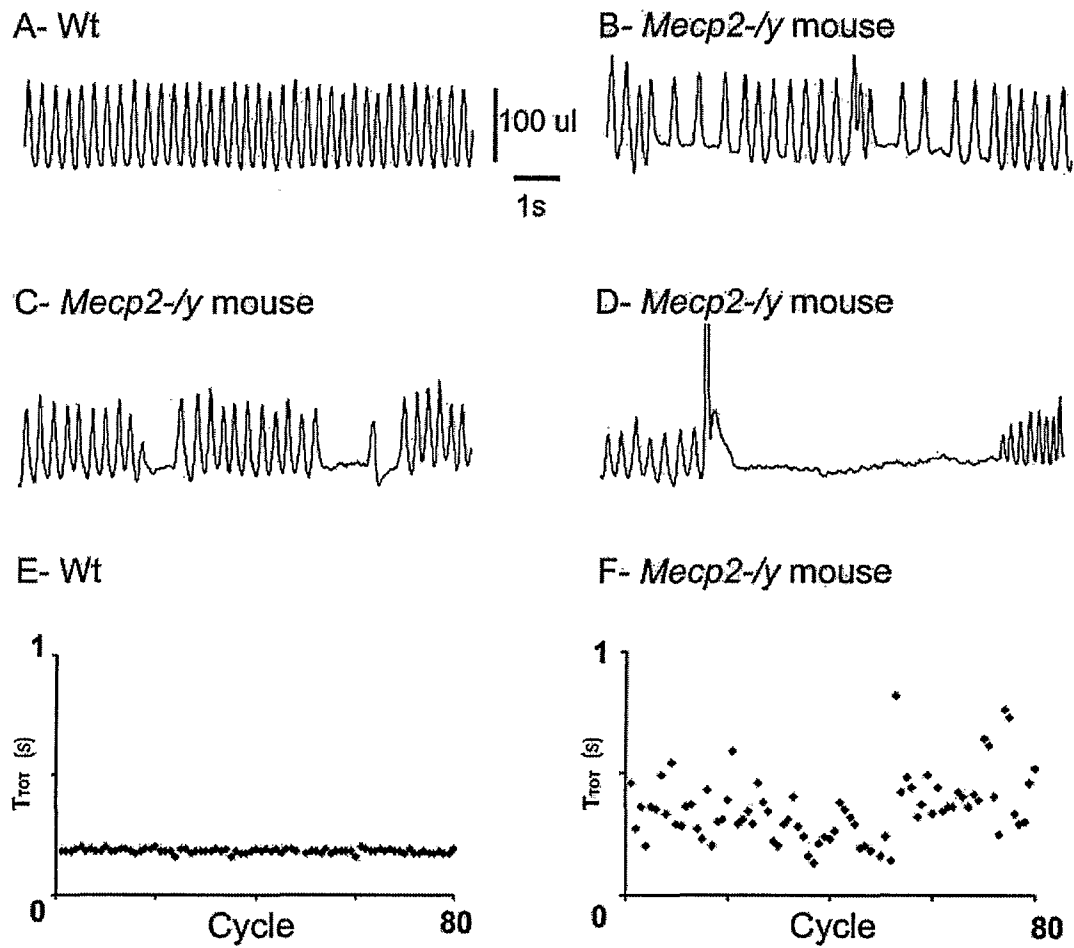


Figure 2

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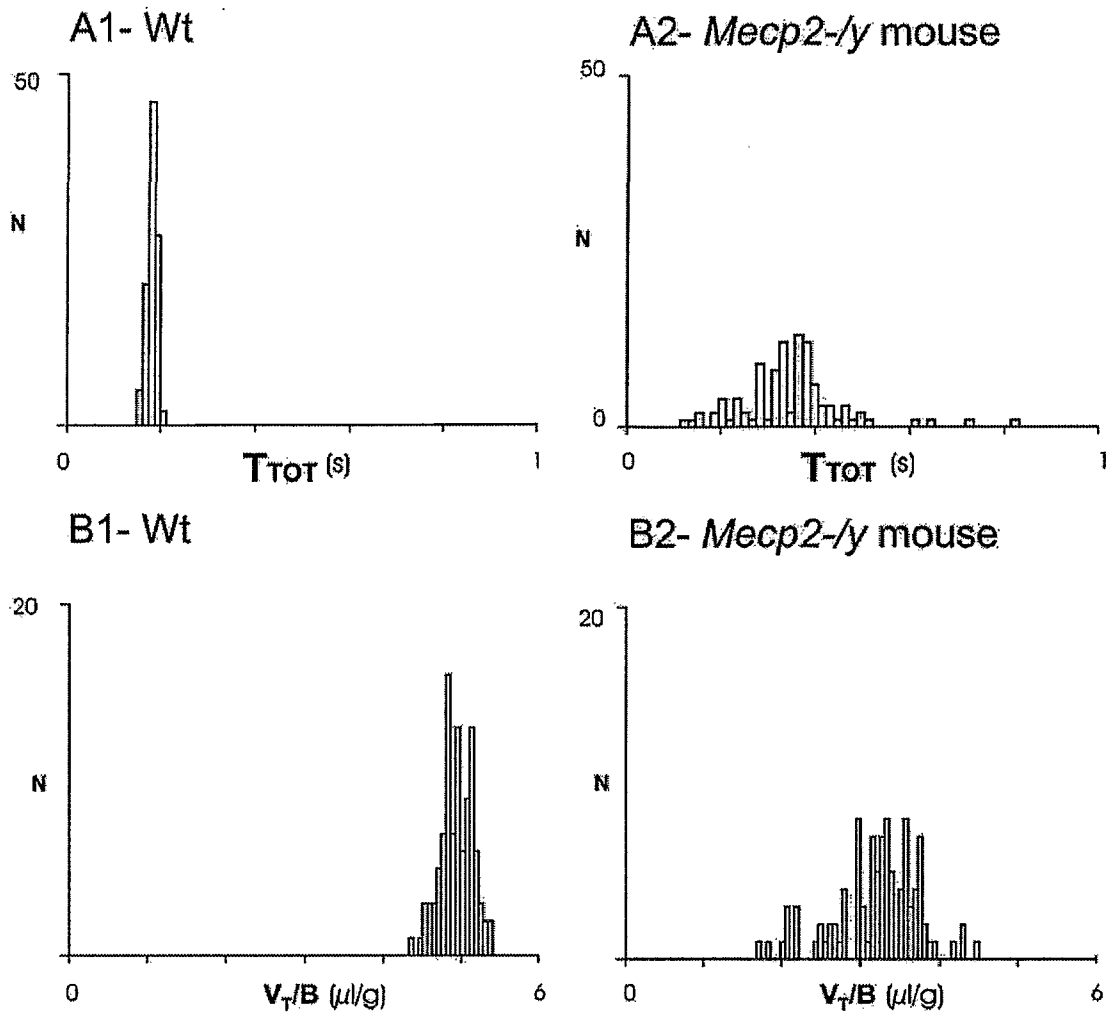


Figure 3

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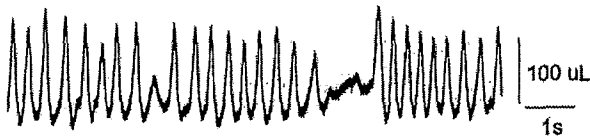
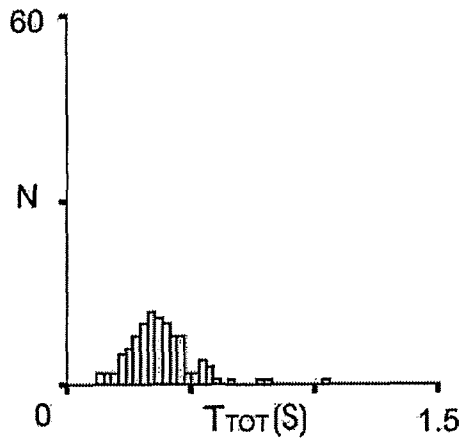
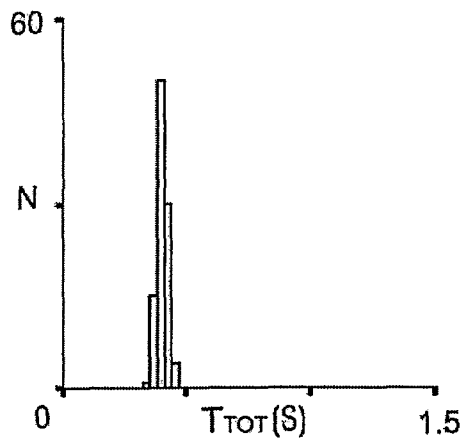
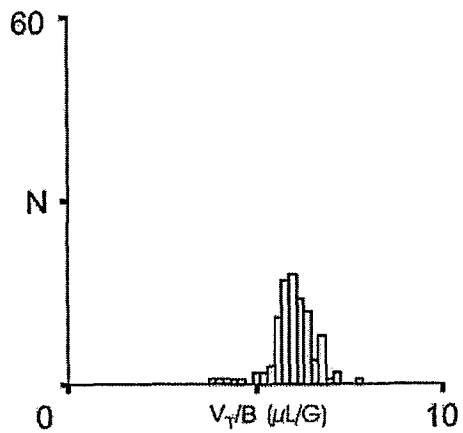
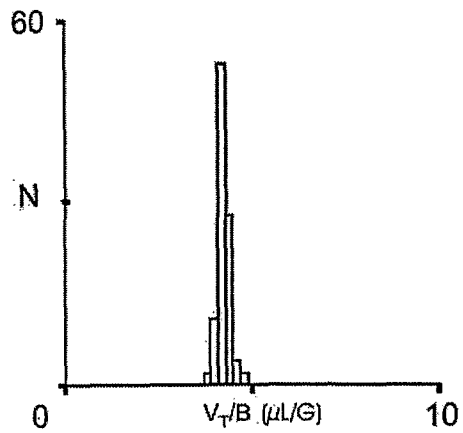
A1- *Mecp2*^{-/-} UnanesthetizedA2- *Mecp2*^{-/-} AnesthetizedB1- *Mecp2*^{-/-} UnanesthetizedB2- *Mecp2*^{-/-} AnesthetizedC1- *Mecp2*^{-/-} UnanesthetizedC2- *Mecp2*^{-/-} Anesthetized

Figure 4

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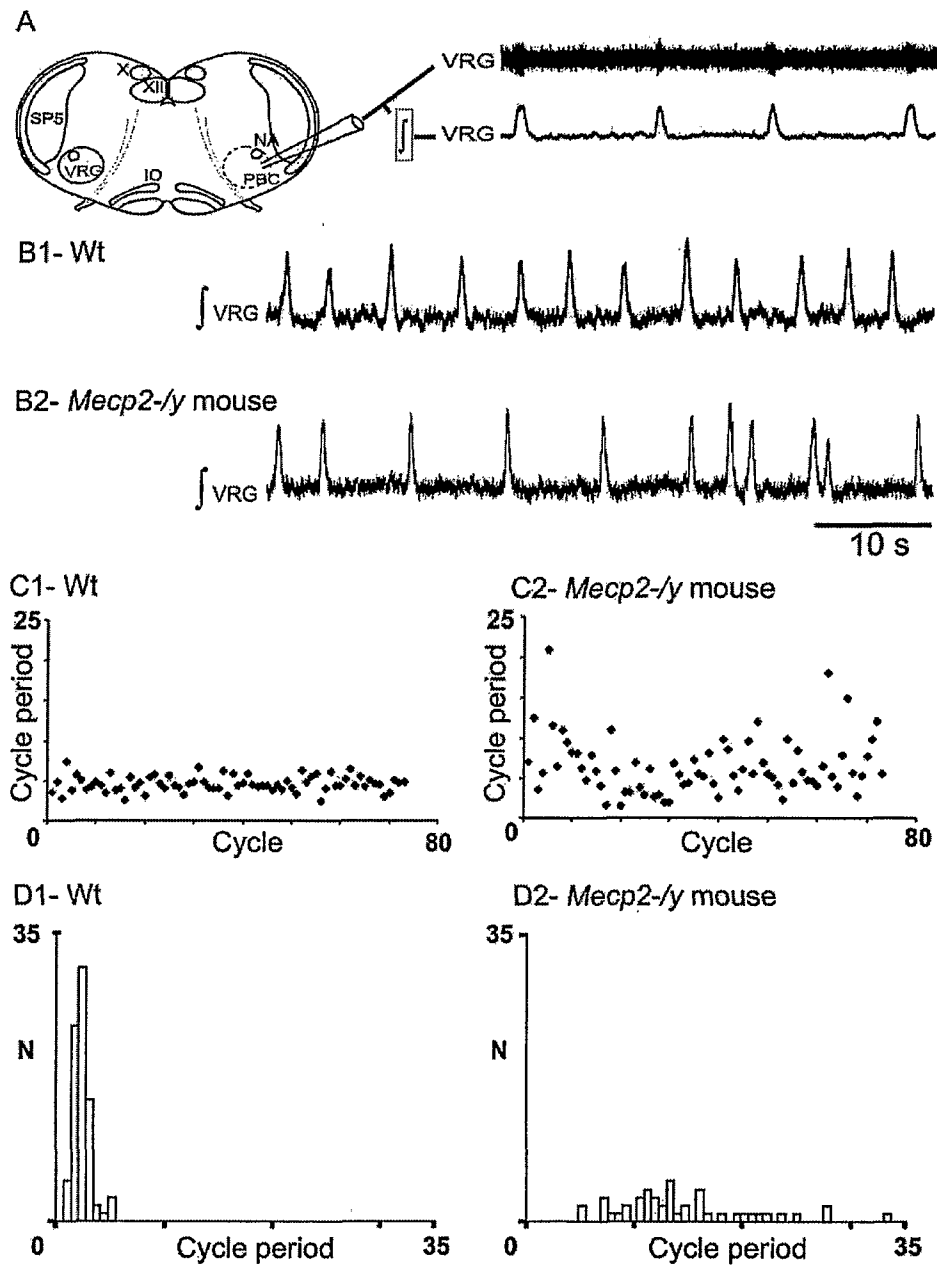


Figure 5

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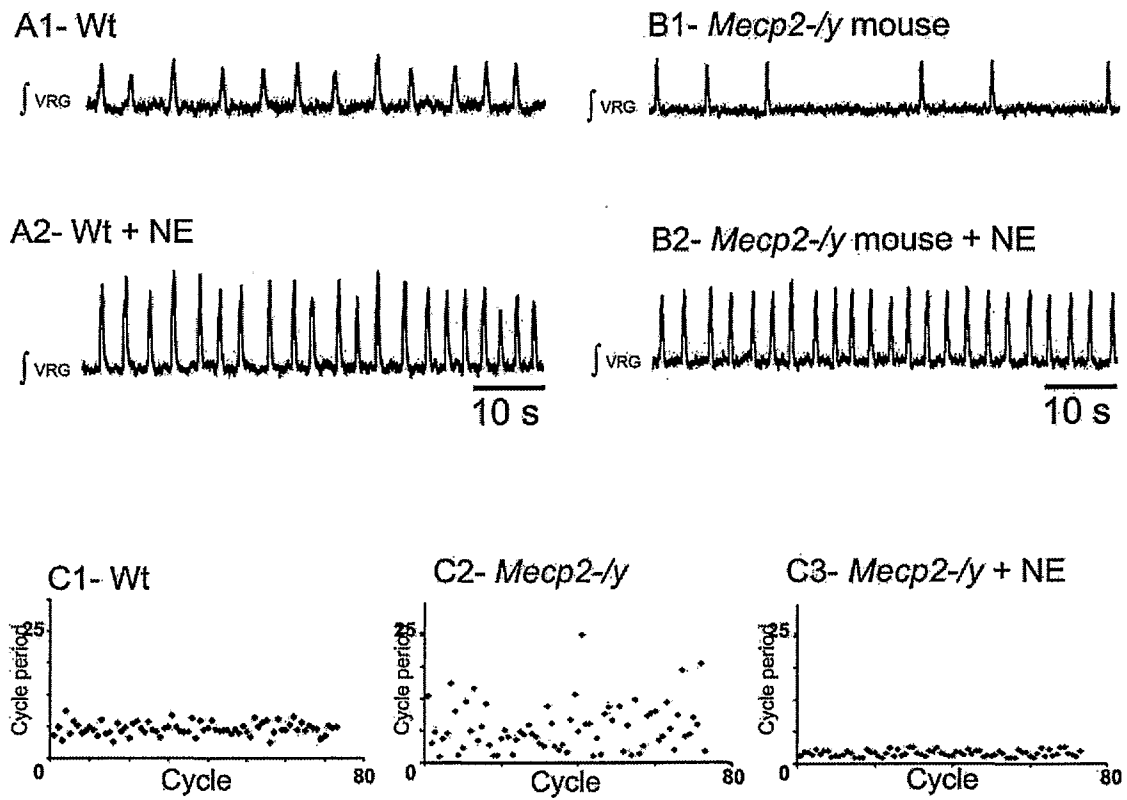


Figure 6

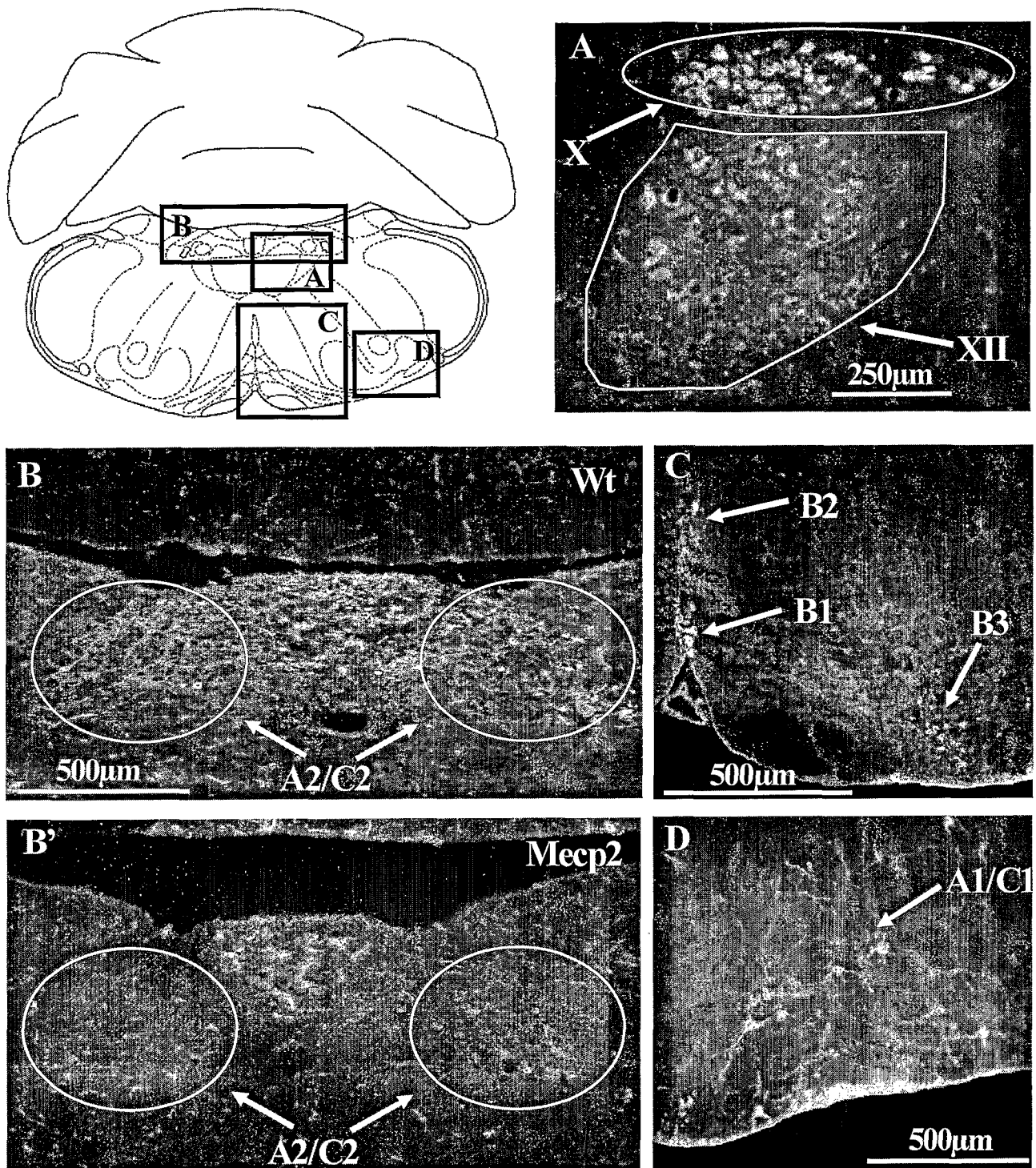


Figure 7

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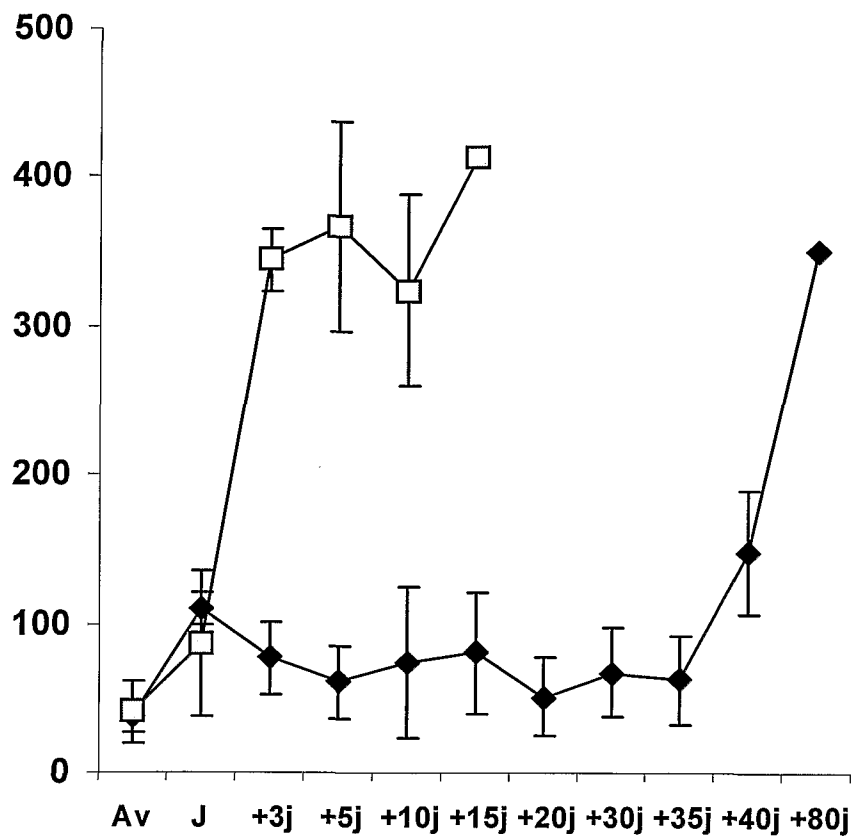


Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/009893

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/55 A61K31/5375 A61K31/137 A61K31/138 A61K31/553
A61K31/336 A61P11/16 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 336 406 A (SOLVAY PHARM BV [NL]) 20 August 2003 (2003-08-20) abstract paragraph [0001] paragraph [0015] claims 1-10,20	1-4
X	WO 2005/020976 A (ELI LILLY AND COMPANY; ALLEN, ALBERT, JOHN; KELSEY, DOUGLAS, KENNETH) 10 March 2005 (2005-03-10) claims 1-4 page 6, line 8 - page 7, line 9 page 15, line 13 - line 16 ----- -/--	1,2

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *O* document referring to an oral disclosure, use, exhibition or other means
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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

12 April 2006

Date of mailing of the international search report

27/04/2006

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Langer, O

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International application No

EP2005/009893

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