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Lu AA21004, a novel multimodal antidepressant, produces regionally selective increases of multiple neurotransmitters—A rat microdialysis and electrophysiology study

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Abstract
The monoaminergic network, including serotonin (5-HT), norepinephrine (NE), and dopamine (DA) pathways, is highly interconnected and has a well-established role in mood disorders. Preclinical research suggests that 5-HT receptor subtypes, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_3, and 5-HT_7 receptors as well as the 5-HT transporter (SERT), may have important roles in treating depression. This study evaluated the neuropharmacological profile of Lu AA21004, a novel multimodal antidepressant combining 5-HT_3 and 5-HT_7 receptor antagonism, 5-HT_{1B} receptor partial agonism, 5-HT_{1A} receptor agonism, and SERT inhibition in recombinant cell lines. Extracellular 5-HT, NE and DA levels were evaluated in the ventral hippocampus (vHC), medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) after acute and subchronic treatment with Lu AA21004 or escitalopram. The acute effects of LuAA21004 on NE and DA neuronal firing were also evaluated in the locus coeruleus (LC) and ventral tegmental area (VTA), respectively. Acute Lu AA21004 dose-dependently increased 5-HT in the vHC, mPFC and NAc. Maximal 5-HT levels in the vHC were higher than those in the mPFC. Furthermore, mPFC 5-HT levels were increased at low SERT occupancy levels. In the vHC and mPFC, but not the NAc, high Lu AA21004 doses increased NE and DA levels. Lu AA21004 slightly decreased LC NE neuronal firing and had no effect on VTA DA firing. Results are discussed in context of occupancy at 5-HT_3, 5-HT_{1B} and 5-HT_{1A} receptors and SERT. In conclusion, Lu AA21004, acting via two pharmacological modalities, 5-HT receptor modulation and SERT inhibition, results in a brain region-dependent increase of multiple neurotransmitter concentrations.

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1. Introduction

Due to their superior safety profile compared with tricyclic antidepressants and monoamine oxidase inhibitors, selective serotonin (5-HT) reuptake inhibitors (SSRIs) have become the most widely prescribed medications for mood disorders. Clinical research suggests that only half of depressed patients respond to an initial trial with SSRIs, and further that only one-third of patients achieve adequate remission from their symptoms with that first trial (Gaynes et al., 2009). Even patients who experience full symptom remission endure a time lag up to several weeks between treatment onset and symptom reduction, and these treatments are associated with side effects. Thus, while available pharmacotherapies for depression are considered moderately effective, there is still room for improvement.

The current zeitgeist in drug development for mood disorders may be described as a trend away from compounds that selectively increase 5-HT concentrations and towards those that non-selectively affect multiple monoaminergic reuptake systems, as in serotonin-norepinephrine (NE) reuptake inhibitors and triple (5-HT, NE and dopamine (DA)) reuptake inhibitors, or those that enhance multiple neurotransmitter systems via a designed multitarget mechanism involving both reuptake inhibition and neurotransmitter receptor effects (Millan, 2009). Concomitant modulation of several serotonergic receptor systems has the potential to improve the effects of current therapies in several ways: enhancement of 5-HT neurotransmission via disinhibition of negative feedback mechanisms, postsynaptic 5-HT receptor stimulation and enhancement of neurotransmitters besides 5-HT via heteroreceptors on non-serotonergic neurons.

Recent hypotheses postulate that the therapeutic lag observed with SSRIs is caused in part by increased activation at somatodendridic 5-HT_{1A} receptors in the dorsal raphe nucleus (DRN), which markedly reduce DRN firing rates and the amount of 5-HT available throughout the brain. Desensitization of 5-HT_{1A} receptors, allowing for greater synaptic 5-HT availability, may therefore be a necessary process for therapeutic efficacy using SSRIs (Blier and Ward, 2003). Thus, compounds combining serotonin reuptake inhibition with high-efficacy 5-HT_{1A} agonism, which may theoretically cause rapid presynaptic 5-HT_{1A} receptor desensitization (Blier and Ward, 2003; Assie et al., 2006), may lead to improved 5-HT availability over short treatment periods. An added benefit of this strategy is that postsynaptic 5-HT_{1A} receptor activation, which is thought to mediate some antidepressant effects (Haddjeri et al., 1998; Blier and Ward, 2003), would also be increased.

5-HT_3 receptors may also play an important role in major depression (Bétry et al., 2011). Localization studies have demonstrated expression of these receptors in limbic regions such as the hippocampus and amygdala as well as the frontal cortex in humans (Bufton et al., 1993) and rodents (Laporte et al., 1992), and preclinical experiments demonstrate that 5-HT_3 receptor antagonists have antidepressant-like effects on their own or potentiate the effects of known antidepressants in models predictive of antidepressant efficacy (Ramamoorthy et al., 2008; Redrobe and Bourin, 1997).

Based on these ideas, a screening program identified Lu AA21004 as a compound of interest, which in addition to being a 5-HT_3 receptor antagonist (Ki = 3.0 nM, h5-HT_3 receptor), 5-HT_{1A} receptor agonist (Ki = 15 nM, h5-HT_{1A} receptor) and SERT inhibitor (IC_{50} = 5.3 nM, hSERT), was also a partial agonist at 5-HT_{1B} receptors (Ki = 33 nM; 55% intrinsic activity h5-HT_{1B} receptor) and an antagonist at 5-HT_7 receptors (Ki = 19 nM) in recombinant cell systems (Bang-Andersen et al., 2011; Mørk et al., 2011).

Here we investigate the effects of acute and subchronic administration of Lu AA21004 or the SSRI escitalopram on extracellular 5-HT, NE and DA levels in the ventral hippocampus (vHC), medial prefrontal cortex (mPFC) and nucleus accumbens (NA) in awake rats and relate the changes in neurotransmitter levels to occupancy levels at 5-HT_3, 5-HT_{1B} and 5-HT_{1A} receptors as well as the SERT. Furthermore, we investigate Lu AA21004's acute effects on NE and DA neuron firing in the locus coeruleus (LC) and ventral tegmental area (VTA), respectively.

2. Experimental procedures

2.1. Animals

Male rats weighing between 250 and 350 g were used. Microdialysis experiments were conducted in Wistar rats from Harlan Labs (Horst, The Netherlands for acute experiments or Livermore, USA for subchronic studies). Electrophysiology and *in vivo* cold receptor occupancy experiments were conducted using Sprague-Dawley rats from Harlan (Gannat, France), or RCC Laboratories (Hyderabad, India), respectively. *Ex vivo* receptor occupancy experiments used either Wistar rats (Livermore, USA) or Sprague-Dawley rats (Charles River, Kingston, NY, USA). All animals were group housed in plastic cages under a 12 h light/dark schedule, with standard temperature and humidity conditions and *ad libitum* access to food and water in the home cages. All experiments were approved by the local institutional animal care committee prior to the start of these studies.

2.2. Chemicals

2.2.1. Drugs

1-[2-(2,4-dimethylphenylsulfanyl)phenyl]piperazine HBr (Lu AA21004) and escitalopram oxalate were synthesized by H. Lundbeck A/S. Chloral hydrate, apomorphine, clonidine, haloperidol, idazoxan, pindolol, and WAY-100635 were purchased from Sigma-Aldrich (St. Quentin Gallavier, France). Lu AA21004 was dissolved in 2-hydroxypropyl-β-cyclodextrin [10–40% (w/v); Roquette America, Inc, Keokuk, IA, USA and St. Quentin Gallavier, France]; all other drugs were dissolved in saline. Specific information on drug administration is noted below for each experiment. All doses are expressed as the mass of the active base.

2.2.2. Radioligands

[^{3}H] n-methyl-2-(2-amino-4-cyanophenylthio)-benzylamine ([^{3}H]DASB; 80 Ci/mmol, 1 mCi/mL) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [^{3}H]GR1257343 (76 Ci/mmol; 0.1 mCi/mL) was purchased from Perkin-Elmer (Boston, MA, USA). [^{3}H]LY278584 (83 Ci/mmol; 0.2 mCi/mL) was purchased from Amersham (Buckinhamshire, UK).

2.3. Microdialysis

2.3.1. Surgical osmotic minipump implantation

Rats chosen for subchronic dosing were anesthetized using isoflurane (2–5%), and an incision was made in the skin of the animal's back. Using a hemostat, a subcutaneous pocket was created parallel

to the spine, where an osmotic minipump (2ML2, Alzet, USA) filled with Lu AA21004 or escitalopram was inserted before the skin was closed, and the rat was returned to its home cage. During the 3 days of treatment via minipumps, the skin surrounding the minipump was massaged daily, preventing skin irritation and stimulating reliable drug exposure.

2.3.2. Stereotaxic placement of microdialysis probes

Animals were anesthetized using isoflurane and mounted within a stereotaxic frame. A small incision was made into the skin over the skull into which a 10% lidocaine solution (w/v) was perfused. Concentric microdialysis probes (Hospal AN 69 membrane; BrainLink, The Netherlands) were stereotaxically inserted into the medial prefrontal cortex (mPFC; anteroposterior (AP): +3.4 mm to bregma, mediolateral (ML): −0.8 mm, dorsoventral (DV): −5.0 mm; 4 mm exposed membrane), nucleus accumbens (NA; AP: +2.0, ML: +1.2 mm, DV −7.9 mm; 2 mm exposed membrane) or ventral hippocampus (vHC; AP: −5.3 mm, ML: −4.8 mm, DV: −8.0 mm; 4 mm exposed membrane). All measurements are relative to bregma according to Paxinos and Watson (1982), with the exception of DV measurements, which were relative to the dura mater. Probes were secured to the skull using dental cement and screws. Rats were singly housed after probe implantation. Surgeries were performed 20–24 h prior to acute experiments or 3 days after osmotic minipump insertion for subchronic experiments.

2.3.3. Microdialysis experiments

On the day of the experiment, implanted probes were connected with flexible PEEK tubing and perfused with artificial CSF containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ at a flow rate of 1.5 μL/min. Microdialysis samples were collected at 20–30 min intervals by an automated fraction collector and stored at −80 °C pending analysis. After each experiment, rats were euthanized and their brains were dissected from the skull, incubated in a 4% paraformaldehyde solution for 3 days, and sectioned coronally to verify probe placement.

2.3.4. Drug administration

For acute drug administration studies, Lu AA21004 was administered at 0.31, 0.63, 1.3, or 2.5, 5, or 10 mg/kg, while escitalopram was administered at 0.49, 1.6 or 4.9 mg/kg. All acute administration was performed subcutaneously (sc) at a 1 mL/kg volume. In subchronic drug administration experiments, animals were treated with vehicle, Lu AA21004, or escitalopram for 3 days via minipumps (delivery rate 5.0 μL/h). Lu AA21004 was administered at 5, 10, 19 or 28 mg/kg day, while escitalopram was administered at 7.5 mg/kg day.

2.3.5. Monoamine determination

Concentrations of 5-HT, NE and DA were determined by high performance liquid chromatography (HPLC) separation with electrochemical or mass spectrometric detection.

2.3.6. Norepinephrine and dopamine

Separation was performed by injecting aliquots (20 μL) onto the HPLC column (Reversed Phase, particle size 3 μm, C18, Thermo BDS Hypersil column, 150 × 2.1 mm, Thermo Scientific, USA) by a refrigerated microsampler, consisting of a syringe pump (Gilson, model 402, France), a multi-column injector (Gilson, model 233 XL, France) and a temperature regulator (Gilson, model 832, France). Chromatographic separation was performed using a mobile phase consisting of a sodium acetate (NaAc) buffer (6.15 g/L) with methanol (MeOH; 2.5% v/v), Titriplex (250 mg/L), octane sulfonic acid (150 mg/L), adjusted to pH=4.1 with glacial acetic acid (isocratic). The mobile phase was pumped at a 0.35 mL/min flow rate by an HPLC pump (Shimadzu, model LC-10AD vp, Japan). NE and DA were detected electrochemically using a potentiostat (Antec Leyden, the Netherlands) fitted with a glassy carbon electrode set at +500 mV vs. Ag/AgCl (Antec Leyden, The Netherlands). Data were analyzed by Chromatography Data System (Shimadzu, class-vp, Japan) software. NE and DA concentrations were quantified by the external standard method.

2.3.7. Serotonin

Aliquots were injected onto the HPLC column as described above. Chromatographic separation was performed using a mobile phase consisting of NaAc (4.1 g/L) with MeOH (3.5% v/v), Titriplex (500 mg/L), heptane sulphonic acid (10 mg/L), and triethylamine (60 μL/L) and adjusted to pH=4.2 with glacial acetic acid (isocratic). Mobile phase was pumped at a flow rate of 0.35 mL/min.

2.4. Receptor occupancy methods

2.4.1. Drug administration and tissue collection

Fractional receptor occupancies at SERT, 5-HT_{1B} and 5-HT_3 were determined via *ex vivo* autoradiography in the slice. In acute dosing experiment 1, rats were treated with 2.5, 5, 10 or 20 mg/kg Lu AA21004 or 0.49, 1.6 or 4.9 mg/kg escitalopram, while animals in a separate acute dosing experiment were administered 0.001, 0.01, 0.1 or 1.0 mg/kg LuAA21004. In subchronic dosing experiments, rats were surgically implanted with subcutaneous minipumps and administered Lu AA21004 or escitalopram for 3 days as noted above.

1 h after treatment in acute experiments or 3 days after minipump implant in subchronic experiments, rats were anesthetized with CO_2 and sacrificed. The brain was harvested, flash frozen on powdered dry ice, and stored at −20 °C. Whole brains were sectioned coronally using a cryostat and mounted on slides. Slices were 20 μm thick, and began at approximately +1.2 mm anterior from bregma for SERT and 5-HT_{1B} receptor occupancy, or −4.8 mm posterior from bregma for 5-HT_3 receptor occupancy determination, respectively. Fig. 1 details the specific brain regions used in each receptor occupancy assay. Slides were stored for at least 24 h at −20 °C before use in autoradiography experiments.

2.4.2. General autoradiography methods

On the day of binding experiments, boxes containing slides were defrosted at room temperature (RT) under a constant stream of air for 30–45 min. Slides were incubated at RT in an assay buffer that included the appropriate tritiated radioligand. After incubation, slides were washed twice in 4 °C assay buffer and briefly dipped in water before being desiccated. Slides were exposed in a Beta imager (Biospace Lab, Paris, France) for 20–24 h. Specific details for each binding assay are noted below. Radioligand concentrations and regions of interest (ROIs) were chosen based on the results of saturation binding and receptor mapping experiments conducted by this laboratory.

2.4.3. SERT occupancy

Slides were incubated for 90 min in assay buffer (50 mM Tris-HCl, 150 mM NaCl and 5 mM KCl, pH 7.4) containing 0.5 nM [^{3}H]DASB. 1 μM escitalopram was used to determine nonspecific binding. After incubation, slides were washed and dried as noted above. Finally, the slides were exposed in the Beta imager for 24 h prior to analysis.

2.4.4. 5-HT_{1B} receptor occupancy

Slides were preincubated for 3 min in a buffer containing 170 mM Tris-HCl, 4 mM $CaCl_2$, 0.1% L-ascorbic acid, pH 7.4 and were then air-dried at RT for 30–45 min. Subsequently, slides were incubated for 60 min in buffer containing 1 nM [^{3}H]GR125743 and 10 μM pargyline. Nonspecific binding was determined using 10 μM SB216641. After incubation, slides were washed and dried as noted above. Finally, the slides were exposed using a Beta imager for 20 h prior to analysis.

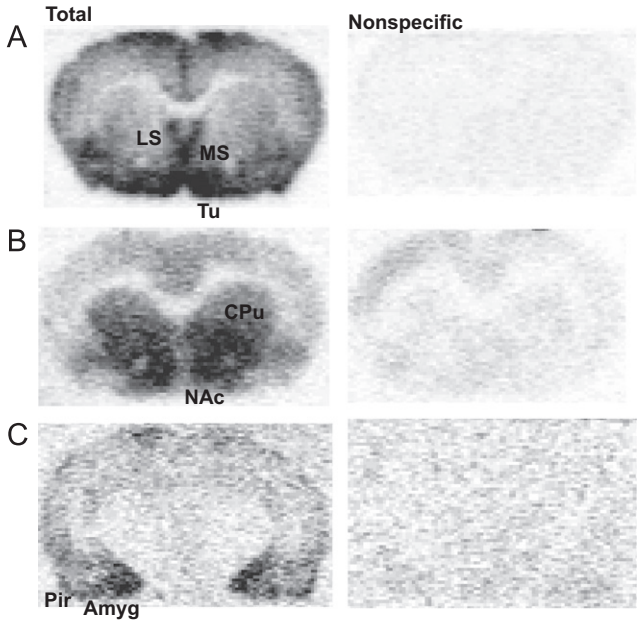

Fig. 1 Regions of interest (ROI) in *ex vivo* autoradiography experiments. Brain Slices representing total (left panels) and non specific binding (right panels) in SERT (A), 5-HT_{1B} (B) and 5-HT_3 (C) autoradiography experiments. The ROI in SERT occupancy experiments included the lateral septum (LS), medial septum (MS) and olfactory tubercle (Tu). 5-HT_{1B} experiments focused on the caudate/putamen (CPu) and nucleus accumbens (NAc), while 5-HT_3 experiments focused on the amygdala (Amyg) and piriform cortex (Pir).

2.4.5. 5-HT_3 receptor occupancy

Slides were preincubated for 5 min in a buffer containing 50 mM Tris-HCl and 150 mM NaCl, pH 7.4 and were then air dried at RT for 30-45 min. Subsequently, slides were incubated for 30 min in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 4 mM $CaCl_2$ and 2 nM [^{3}H]LY278584 (83 Ci/mmol). Nonspecific binding was determined using 10 μM ondansetron. After being washed and dried as noted above, the slides were exposed using a Beta imager for 24 h prior to analysis.

2.4.6. 5-HT_{1A} receptor occupancy

5-HT_{1A} receptor occupancy was determined via *in vivo* cold competition, using liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection. Rats were treated with Lu AA21004 at 2.5, 5, 10 or 20 mg/kg or vehicle sc, or 3 mg/kg pindolol iv. All drugs were injected at 2 mL/kg. 10 min after pindolol or 30 min after Lu AA21004, animals were treated with the tracer compound WAY-100635 via lateral tail vein at a constant flow-rate as an i.v. bolus dose (3 μg/kg, 0.5 mL/kg).

Rats were sacrificed 30 min after the tracer dose and the brain was separated from the skull without damaging the brain structure and placed on an absorbent wipe. The brain was washed with cold water and kept on a Whatman filter, which was placed on a closed Petri dish containing crushed ice. The frontal cortex and cerebellum (non-specific binding) were isolated and stored below -50 °C until analysis.

2.4.7. Analysis of unlabeled WAY-100635 levels

Pre-weighed brain tissue samples were placed in conical 1.5 mL centrifuge tubes to which 4 volumes (w/v) of acetonitrile containing 0.1% formic acid was added. Samples were homogenized using an ultrasonic dismembrator probe, vortexed and centrifuged for 15 min at 16,000*g*. WAY-100635 concentration in supernatant was measured using a validated LC-MS/MS method.

2.5. Electrophysiology methods

2.5.1. Surgical methods

Rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and mounted in a stereotaxic frame. A lateral tail vein was cannulated with a 24-guage catheter for i.v. drug administration. Extracellular recordings were performed with single-barreled glass micropipettes prefilled with fiberglass filaments. The tip was broken back to 2-4 μm and filled with 0.5 M NaAc saturated with blue Chicago dye.

For DA neuron recordings, the electrode was placed stereotaxically in the VTA (AP: −4.8 to −5.2; ML: 0.5 to 0.8; DV: −7.5 to −8.5, relative to bregma). DA neurons were identified as previously reported (Bunney and Aghajanian, 1977; White and Wang, 1983) and only neurons having action potential widths ≥1.1 ms from start to negative trough (Ungless et al., 2004) were recorded.

LC NE neurons were recorded at the following stereotaxic coordinates: (AP: −0.7, ML: 1.1 to 1.4; DV: −4.5 to −6 measurements relative to lambda). NE neurons were identified by their regular firing rate (1-5 Hz), long duration (0.8-1.2 ms), positive action potentials and their characteristic burst discharge in response to a nociceptive pinch of the contralateral hind paw (Aghajanian, 1978).

2.5.2. Drug administration

Once a neuron was identified, baseline firing rate was established over 2-3 min. Subsequently, drugs were administered with a delay of approximately 80 s between each i.v. injection. First, saline was administered, followed by successive 2.5 mg/kg Lu AA21004 injections. In cases where Lu AA21004 treatment failed to fully suppress firing, it was followed by pharmacological treatments known to fully suppress firing and finally to recover firing in putative DA/NE cells. In DA experiments, Lu AA21004 treatment was followed by an injection of apomorphine (50 μg/kg) in order to fully suppress firing, and haloperidol (0.2 mg/kg) to recover from this suppression. Similarly, in NE experiments, clonidine (5 μg/kg) was used to suppress firing rates and idazoxan (0.5 mg/kg) was used to recover from this suppression.

2.6. Data analysis and statistical methods

2.6.1. Microdialysis

Three to four consecutive pre-treatment microdialysis samples with <50% variation were used as baseline and their mean concentration was set at 100%. Drug effects were expressed as percentages of basal level (mean ± SEM) within the same subject. Raw extracellular neurotransmitter concentrations (in fmol/sample) were tested for outliers using Grubb's test. For acute experiments, data for each individual was normalized to its own baseline, and expressed as a percentage of basal concentration. Each individual's area under the curve was calculated from the injection time to the end of the experiment (0-160 min) using the trapezoid method, and log transformed. Subchronic experiments used raw basal concentration values as a dependent measure. Significance testing was performed using one way ANOVA, followed by Neuman-Keuls post-hoc tests when appropriate.

2.6.2. *Ex vivo* autoradiography

Surface radioactivity (expressed as cpm/mm^2) was measured using Beta vision+ software version 2.0 (Biospace Lab, Paris, France). Radioactivity was quantified from a ROI defined *a priori* for each binding assay and remained consistent across each slice of tissue (Fig. 1). Specific binding was determined by subtracting nonspecific binding from total binding. Specific binding for each brain was normalized to the average specific-bound radioactivity from vehicle-treated individuals and expressed as a percentage of vehicle binding. These percentages were subtracted from 100% to obtain percent receptor occupancy. When appropriate, ED_{50} analysis was conducted using Graphpad Prism version 4.02 (GraphPad software,

San Diego, CA, USA). Briefly, a log transformation was performed on Lu AA21004 dose. A non-linear regression was then performed on occupancy values using a sigmoidal dose response curve. The top and bottom values were constrained to 100 and 0, respectively, while the Hill coefficient was not constrained.

2.6.3. Cold *in vivo* occupancy

Receptor occupancy calculations were made for each animal using the following equation:

$$\%\text{Occupancy} = 100\{1-[(\text{treatment}-1)/(\text{control}-1)]\}$$

The variable 'treatment' represents the ratio of WAY-100635 concentrations measured in the frontal cortex to those measured in the cerebellum in animals pre-treated with Lu AA21004, pindolol or vehicle. The variable 'control' represents the average ratio of WAY-100635 levels measured in the frontal cortex to that measured in the cerebellum for the vehicle group.

2.6.4. Electrophysiology

Analysis of pharmacological effects on neuronal firing was conducted on firing rate data that was normalized to baseline and expressed as a percentage of basal values. These data were analyzed using two-way repeated measures ANOVA with Dunnett's post-hoc tests when appropriate.

3. Results

3.1. Microdialysis

3.1.1. Acute dosing

In the vHC, acute escitalopram significantly increased extracellular 5-HT compared to vehicle at all doses tested (Fig. 2; $F(3, 24)=28.93$, $p<0.0001$). Similarly, Lu AA21004 produced a significant increase of extracellular 5-HT compared with vehicle at all doses (Fig. 3A; $F(3, 18)=31.35$, $p<0.001$). In addition, Lu AA21004 significantly increased vHC NE (Fig. 3B; $F(3,17)=8.24$, $p<0.01$) and DA levels (Fig. 3C; $F(3,17)=4.22$, $p<0.05$) compared to vehicle at the 10 mg/kg dose.

In the NAc, acute treatment with Lu AA21004 significantly increased extracellular 5-HT levels at all doses tested (Fig. 4A; $F(3,18)=31.35$, $p<0.0001$), but did not significantly affect NE (Fig. 4B; $F(3,14)=1.19$, n.s.) or DA levels (Fig. 4C; $F(3,15)=1.19$, n.s.) compared to vehicle.

In the mPFC, acute Lu AA21004 produced significant increases in extracellular 5-HT levels at doses from 0.31 to 5 mg/kg (Fig. 5A; $F(4,25)=11.9$, $p<0.0001$) and at 10 mg/kg (Fig. 5B; $F(3,16)=15.3$, $p<0.001$) compared to vehicle control. Moreover, the effect of Lu AA21004 on extracellular 5-HT was dose dependent over this range. Lu AA21004 significantly increased NE levels at the 5 and 10 mg/kg doses (Fig. 6A; $F(3,16)=9.015$, $p<0.001$), while DA was significantly increased (Fig. 6B; $F(3,19)=4.0$, $p<0.05$) only at the 10 mg/kg dose compared to vehicle.

3.1.2. Subchronic (3-day) dosing

In the vHC, Lu AA21004 significantly increased 5-HT levels at 19 and 28 mg/kg day, as did 7.5 mg/kg day escitalopram (Fig. 7A; $F(5, 47)=48.87$, $p<0.001$) compared to vehicle. Lu AA21004 increased extracellular 5-HT to a significantly greater extent than escitalopram at the 19 and 28 mg/kg day doses, despite similar SERT occupancies. In addition, LuAA21004 significantly increased basal NE levels in the vHC at 28 mg/kg day (Fig. 7B; $F(5,40)=4.46$, $p<0.01$), while escitalopram did not. Neither drug affected extracellular DA in this brain region (Fig. 7C; $F(5,44)=2.06$, n.s.) compared to vehicle.

In the mPFC, subchronic Lu AA21004 administration produced dose-dependent increases in extracellular 5-HT (Fig. 8A; $F(5,49=36.29$, $p<0.001$) compared to vehicle. 7.5 mg/kg day escitalopram also significantly increased 5-HT levels, but 28 mg/kg day Lu AAA21004 increased 5-HT levels to a significantly greater extent. NE levels were significantly increased by 28 mg/kg day of Lu AA21004, but not by escitalopram (Fig. 8B; $F(5,44)=6.46$, $p<0.001$), compared to vehicle control. Extracellular DA was significantly increased by both escitalopram and the 28 mg/kg day dose of Lu AA21004 (Fig. 8C; $F(5,35)=5.71$, $p<0.001$) compared to vehicle control.

3.2. Receptor occupancy

Lu AA21004, 2.5–20 mg/kg produced dose-dependent occupancy at the SERT (85–98%) and 5-HT_{1A} (6.8–44%), and 5-HT_{1B} 45-78%) receptors (Table 1). Occupancy at the 5-HT_3 receptor ranged from 32% to 100% over a dose range from 0.001 to

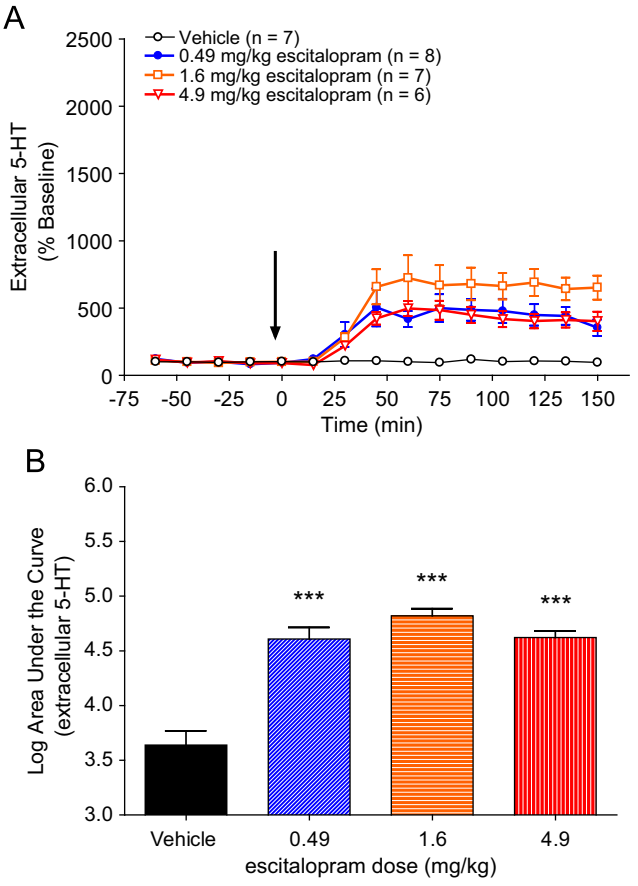

Fig. 2 Escitalopram elevates ventral hippocampus extracellular serotonin. Acute escitalopram increased 5-HT in the vHC. (A) Symbols represent extracellular 5-HT concentration (mean ± SEM) normalized to baseline. (B) Bars represent average log transformed area under the curve (mean ± SEM). Asterisks show statistically significant differences from vehicle (***$p<0.0001$).

1.0 mg/kg (Table 2), resulting in ED_{50}=0.0043 mg/kg (95% confidence interval: 0.0016–0.011).

Subchronic administration of Lu AA21004 (5–28 mg/kg day) also led to dose-dependent increases in SERT occupancy (43–95%; Table 3). For comparison, SERT occupancy was 92% with subchronic dosing of escitalopram 7.5 mg/kg day.

3.3. NE and DA neuronal firing in LC and VTA

At high i.v. doses of 2.5, 5.0, 7.5 and 10.0 mg/kg, Lu AA21004 had no effect on DA neuronal firing in the VTA (Fig. 9A and B; $F(4,34)=1.07$, n.s.) compared to vehicle control. These doses produced a small but statistically significant inhibition of NE neuronal firing in the LC, with a maximal inhibition of approximately 20% (Fig. 9C and D; $F(4,28)=9.219$, $p<0.0001$) compared to vehicle.

4. Discussion

This is the first comprehensive neurochemical and electrophysiological evaluation of the novel antidepressant Lu AA21004. Our results demonstrate that after acute or subchronic 3-day administration, Lu AA21004 caused robust, dose-dependent increases extracellular 5-HT levels, as well as more modest effects on NE, and DA, in mood disorder-relevant brain regions such as the mPFC and vHC. Extracellular 5-HT levels were also significantly increased after acute administration in the NAc, while NE and DA levels were not affected. Interestingly, the magnitude of Lu AA21004's effects on neurotransmitter levels was region-specific, with 5-HT concentrations after acute or subchronic Lu AA21004 being markedly larger at high doses in the vHC than in the mPFC. Moreover, subchronic (three day) Lu AA21004 increased 5-HT levels to a significantly greater extent than the SSRI escitalopram in the mPFC and vHC, and significantly elevated NE in these regions while the SSRI escitalopram did not. Importantly, these differing neurochemical profiles were found despite similar occupancy levels at the SERT, suggesting that Lu AA21004's effects at 5-HT_{1A}, 5-HT_{1B}, 5-HT_3 and/or 5-HT_7 receptors play an important role in this compound's neurochemical effects. Finally, subchronic Lu AA21004 and escitalopram both produced a statistically significant increase of DA in the mPFC. Thus, Lu AA21004 is a multimodal compound (Nutt, 2009), mediating its pharmacological activity via receptor modulation and SERT inhibition.

4.1. Lu AA21004 increases 5-HT concentrations at low levels of SERT occupancy

It is thought that 5-HT clearance within the brain is not significantly altered until nearly all functioning SERT sites have been inhibited. This idea is supported by preclinical data demonstrating reduced 5-HT clearance only in animals with ≥90% of SERT destroyed (Montanez et al., 2003), and is in line with human *in vivo* SERT studies showing ≥80% occupancy is achieved at therapeutic SSRI doses (Meyer et al., 2004; Suhara et al., 2003). Interestingly, acute Lu AA21004 elevated mPFC 5-HT levels at acute doses as low as 0.31 mg/kg, corresponding to only 50% SERT occupancy (ED_{50}=0.4 mg/kg; Mørk et al., 2011), perhaps suggesting that Lu AA21004 increased 5-HT levels by more complex mechanisms than simple reduction of 5-HT clearance. Lu AA21004 occupies 90% of 5-HT_3 receptors at this dose in rodents, thus it is plausible that Lu AA21004's 5-HT_3 antagonist effects are acting in concert with SERT inhibition to potentiate 5-HT

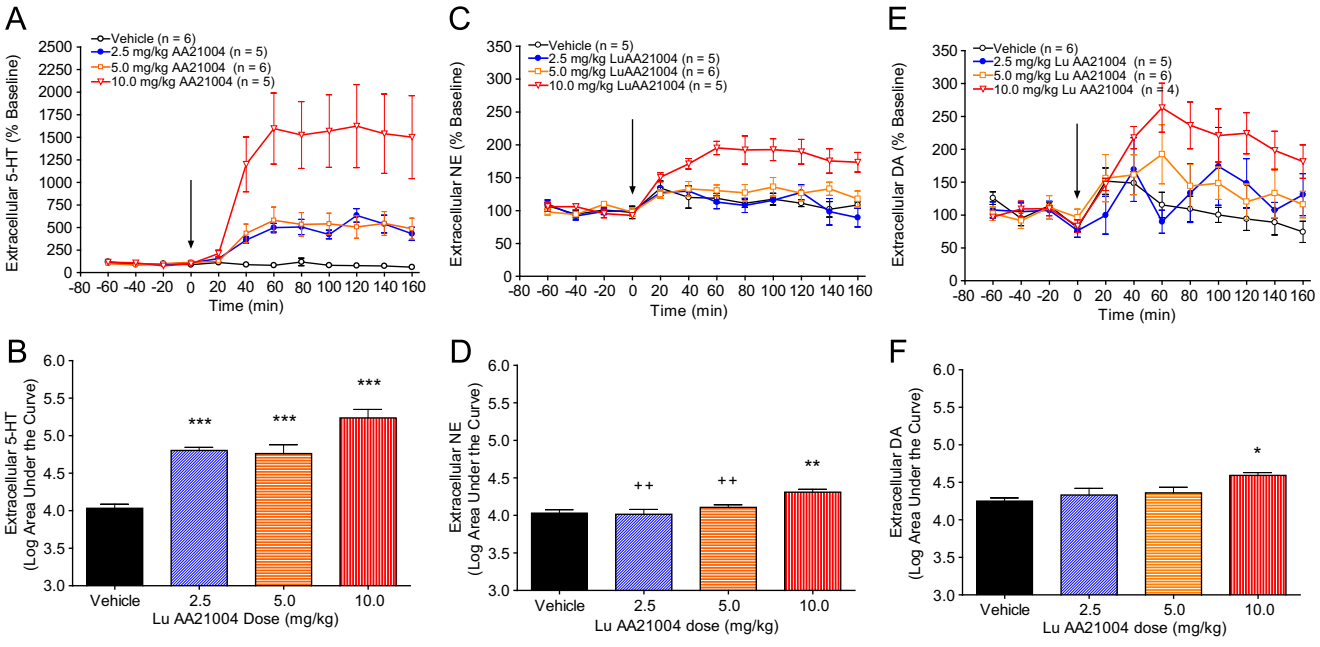

Fig. 3 Acute Lu AA21004 elevates ventral hippocampus extracellular serotonin, norepinephrine and dopamine. Lu AA21004 elevated extracellular 5-HT at all doses, while NE and DA were elevated only at 10.0 mg/kg. In panels (A), (C) and (E), symbols represent extracellular concentrations of serotonin, norepinephrine or dopamine, respectively (mean±SEM), normalized to baseline. Bars in panels (B), (D) and (F) represent log transformed area under the curve for serotonin, norepinephrine or dopamine, respectively (mean±SEM). Asterisks show statistically significant differences from vehicle (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (A) is reprinted with permission from Bang-Andersen et al. (2011). Copyright 2011. American Chemical Society.

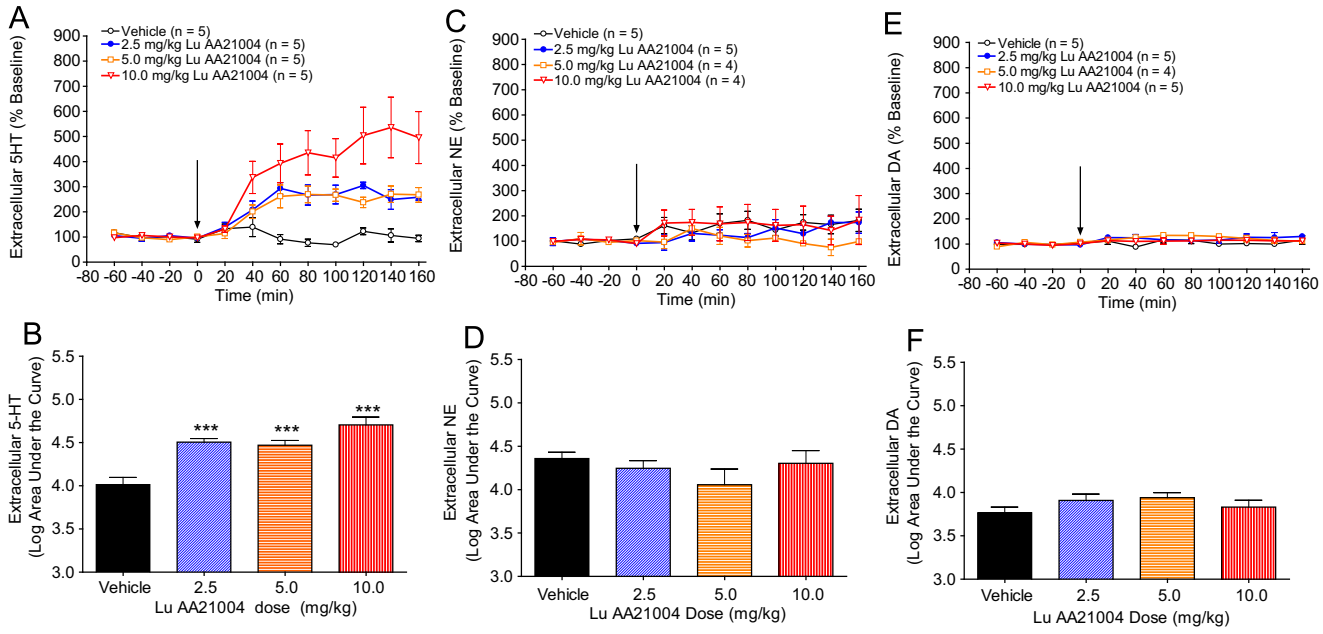

Fig. 4 Acute Lu AA21004 increases serotonin, but not norepinephrine or dopamine in the nucleus accumbens (NAc). Acute Lu AA21004 increased serotonin at all doses, while norepinephrine and dopamine were not affected. In panels (A), (C) and (E), symbols represent extracellular concentrations of serotonin, norepinephrine or dopamine, respectively (mean ± SEM), normalized to baseline. Bars in panels (B), (D) and (F) represent log transformed area under the curve for serotonin, norepinephrine, or dopamine, respectively (mean ± SEM). Asterisks represent statistically significant differences from vehicle ($*p<0.05$, $**p<0.01$, $***p<0.001$).

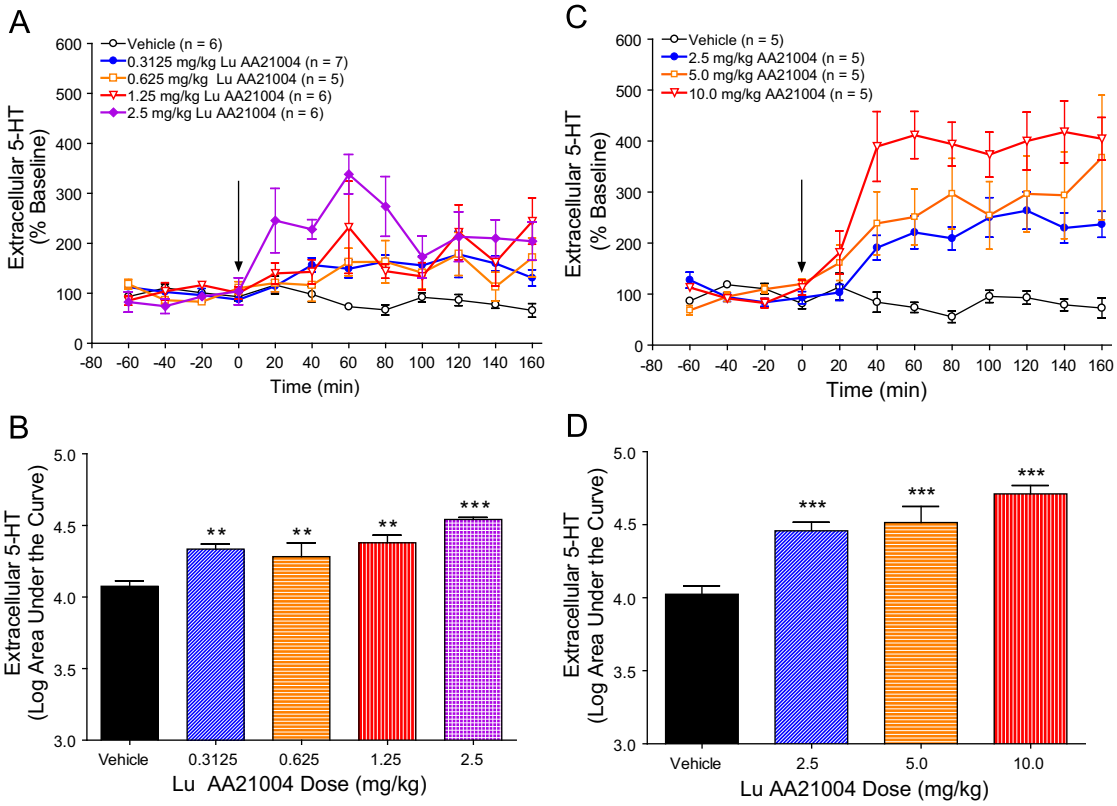

Fig. 5 Acute Lu AA21004 elevates medial prefrontal cortex (mPFC) extracellular serotonin over a broad range of doses. All doses of Lu AA21004 significantly elevated 5-HT compared to vehicle. In panels (A) and (C), symbols represent extracellular concentrations of serotonin (mean ± SEM) normalized to baseline. Bars in panels (B) and (D) represent log transformed area under the curve for serotonin (mean ± SEM). Asterisks represent statistically significant differences from vehicle ($*p<0.05$, $**p<0.01$, $***p<0.001$).

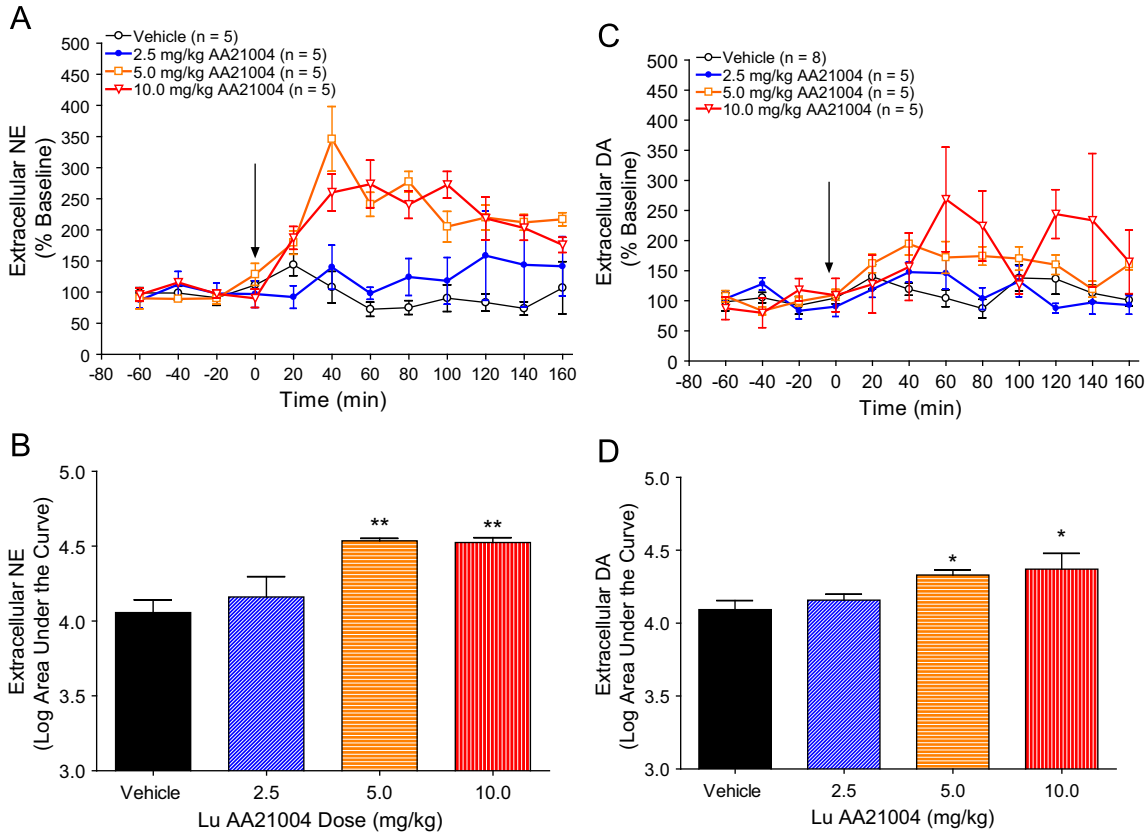

Fig. 6 Acute Lu AA21004 increases medial prefrontal cortex (mPFC) norepinephrine and dopamine release. Lu AA21004 significantly increased extracellular norepinephrine and dopamine at the highest dose tested. In panels (A) and (C), symbols represent extracellular concentrations of norepinephrine, or dopamine, respectively (mean ± SEM), normalized to baseline. Bars in panels (B) and (D) represent log transformed area under the curve for norepinephrine or dopamine, respectively (mean ± SEM). Asterisks represent statistically significant differences from vehicle (∗$p<0.05$, ∗∗$p<0.01$, ∗∗∗$p<0.001$).

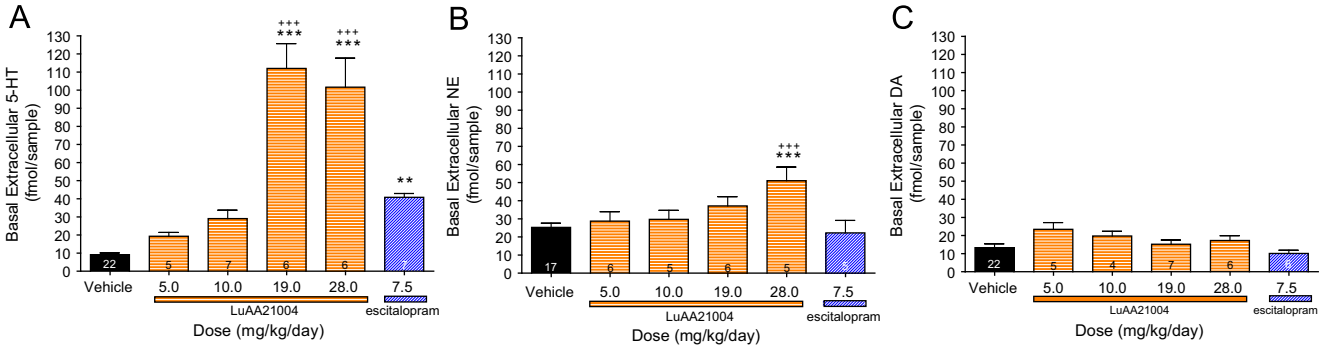

Fig. 7 Subchronic Lu AA21004 and escitalopram differentially regulate basal serotonin, norepinephrine, and dopamine concentrations in the ventral hippocampus (vHC). Subchronic Lu AA21004 and escitalopram significantly elevated serotonin, but Lu AA21004 increased 5-HT to a greater extent than escitalopram at high doses (A). Lu AA21004, but not escitalopram, elevated basal norepinephrine concentrations (B). Neither compound altered basal dopamine release in this region. Asterisks represent significant differences from vehicle (∗$p<0.05$, ∗∗$p<0.01$, ∗∗∗$p<0.001$), while plus signs indicate statistically significant differences between Lu AA21004 and escitalopram (+ $p<0.05$; ++ $p<0.01$; +++ $p<0.001$).

output. This notion is supported by recent results that show citalopram-induced increases in extracellular 5-HT were potentiated by the 5-HT_3 antagonist ondansetron (Mørk et al., 2012). In addition, several reports indicate that 5-HT_3 antagonism augments the antidepressant-like effects of SSRIs (Redrobe and Bourin, 1997; Ramamoorthy et al., 2008).

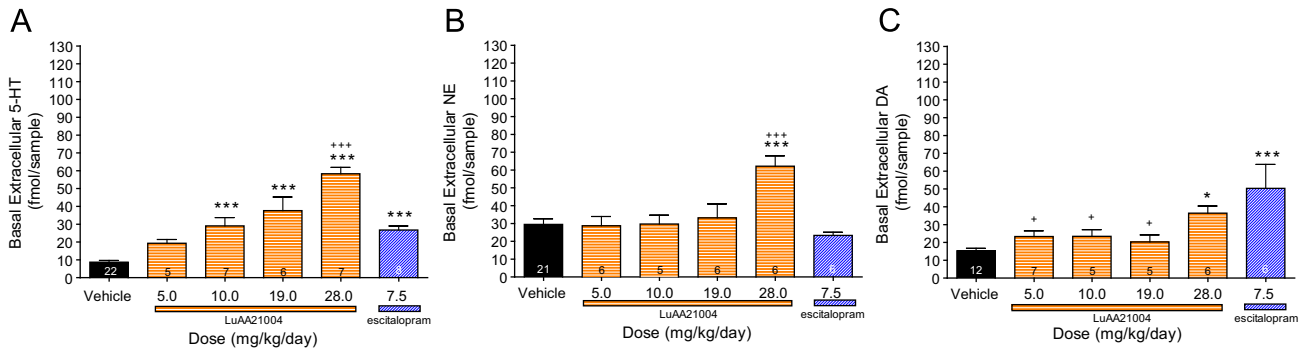

Fig. 8 Subchronic Lu AA21004 and escitalopram differentially regulate basal serotonin, norepinephrine and dopamine concentrations in the medial prefrontal cortex (mPFC). Subchronic Lu AA21004 and escitalopram significantly elevated serotonin, but Lu AA21004 increased 5-HT to a greater extent than escitalopram (A). Lu AA21004, but not escitalopram, elevated basal norepinephrine concentrations (B). Both Lu AA21004 and escitalopram significantly elevated dopamine (C). Asterisks represent significant differences from vehicle (*$p<0.05$, **$p<0.01$, ***$p<0.001$), while plus signs indicate statistically significant differences between Lu AA21004 and escitalopram (+ $p<0.05$; ++ $p<0.01$; +++ $p<0.001$).

Table 1 Acute effects of LuAA21004 administration on 5-HT transporter, 5-HT_{1B} and 5-HT_{1A} receptor occupancy (mean ± SEM). Entries of N/A (not applicable) were entered for escitalopram under 5-HT_{1A} and 5-HT_{1B} receptors, where this drug has no affinity.

Compound	Dose (mg/kg)	SERT occupancy (%)	5-HT_{1A} receptor occupancy (%)	5-HT_{1B} receptor occupancy (%)
Lu AA21004	2.5	85 ± 1.9	6.8 ± 6.8	45 ± 4.8
	5.0	92 ± 0.81	28 ± 3.2	62 ± 5.6
	10.0	94 ± 0.79	35 ± 2.9	79 ± 0.58
	20.0	98 ± 0.65	44 ± 3.8	78 ± 2.2
Escitalopram	0.49	87 ± 4.0	N/A	N/A
	1.6	95 ± 0.98	N/A	N/A
	4.9	100 ± 0.48	N/A	N/A

Table 2 Effects of acute Lu AA21004 administration on 5-HT_3 receptor occupancy (mean ± SEM).

Lu AA21004 dose (mg/kg)	5-HT_3 receptor occupancy (%)
0.0010	32 ± 13
0.010	56 ± 12
0.10	94 ± 3.2
1.0	100 ± 0.61

Table 3 Subchronic effects of Lu AA21004 or escitalopram on 5-HT transporter (SERT) occupancy (mean ± SEM).

Dose (mg/kg day)	SERT occupancy (%)
LuAA21004 5.0	43 ± 8.7
LuAA21004 10.0	57 ± 7.5
LuAA21004 19.0	89 ± 5.3
LuAA21004 28.0	98 ± 0.6
Escitalopram 7.5	92 ± 1.3

4.2. Large increases in vHC extracellular 5-HT compared to mPFC and escitalopram may involve 5-HT_{1B} receptor-mediated effects

5-HT output in terminal regions is controlled in part by 5-HT_{1B} receptor activation, which acts to reduce extracellular 5-HT. In support of this theoretical role, extracellular 5-HT is reduced after 5-HT_{1B} receptor activation (Bosker et al., 1995), or increased after 5-HT_{1B} antagonism (Cremers et al., 2000). Malagie et al. (2001) showed increased 5-HT levels in response to SSRI treatment in the vHC of 5-HT_{1B} receptor knock-out mice compared to wild-type. Interestingly, there was no significant difference in 5-HT levels between 5-HT_{1B} receptor KO and wild-type in the frontal cortex, a phenomenon the authors attributed to low 5-HT_{1B} expression in the frontal cortex of wild-type mice. This result is particularly relevant in light of the greatly increased levels of 5-HT noted in the vHC compared to mPFC in response to high doses of acute or subchronic Lu AA21004. As in mice, 5-HT_{1B} receptor expression is lower in the rat mPFC compared to the vHC (Bruinvels et al., 1993). This, in combination with the 80% 5-HT_{1B} receptor occupancy achieved at the high dose of Lu AA21004, makes it

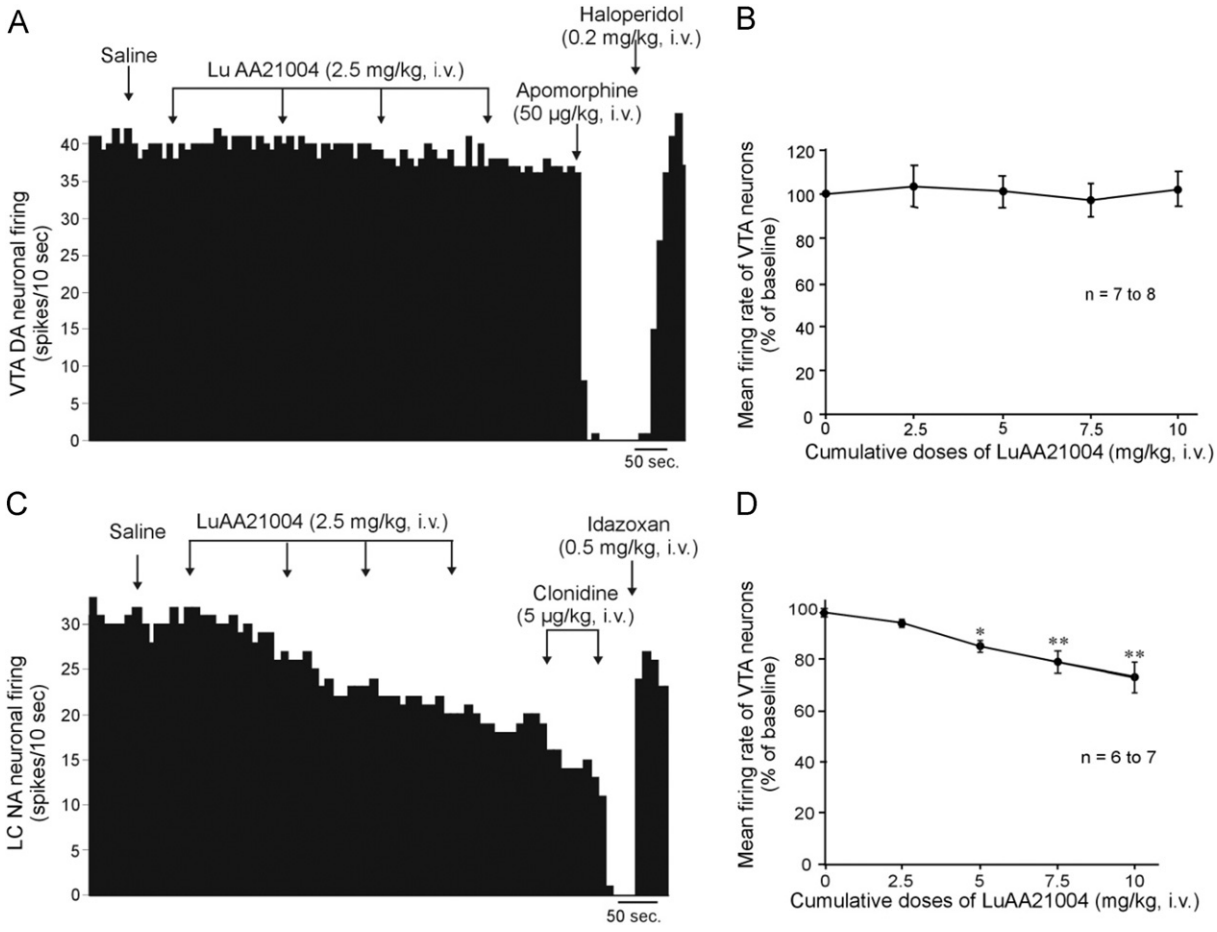

Fig. 9 Acute Lu AA21004 has no effect on ventral tegmental area (VTA) dopamine (DA) neurons, but mildly inhibits locus coerulus (LC) norepinephrine (NE) cells. (A) Integrated firing rate histogram of a presumed VTA DA neuron showing its response to cumulative doses of Lu AA21004 (2.5–10 mg/kg, i.v.). Apomorphine induced full inhibition that was reversed by haloperidol. (B) Cumulative Lu AA21004 doses caused no significant suppression of VTA DA neurons. (C) Integrated firing rate histogram of a presumed LC NA neuron. The combination of Lu AA21004 and clonidine induced full inhibition, which was reversed by idazoxan. (D) Cumulative doses of 2.5–10 mg/kg Lu AA21004 (i.v.) caused a limited but significant inhibition of LC NE neural firing. For both (B) and (D), symbols represent the percent inhibition of cell firing (mean $\pm$ SEM). Asterisks represent statistically significant differences from baseline ($*p<0.05$).

plausible that the region-specific augmentation of extracellular 5-HT is in part due to the action of Lu AA21004 at 5-HT_{1B} receptors.

In recombinant cell lines, Lu AA21004 acts as a partial agonist at the 5-HT_{1B} receptor. However, in the face of this compound's 5-HT reuptake inhibition properties, it may be that Lu AA21004 acts as an antagonist *in vivo* by competing with endogenous 5-HT for the 5-HT_{1B} receptor site. While this idea is speculative, it may explain the 5-HT_{1B} receptor antagonist-like effect seen at high doses of Lu AA21004.

Antagonism at 5-HT_7 receptors may also be involved in Lu AA21004's effects on extracellular 5-HT. Lu AA21004 is an antagonist at 5-HT_7 receptors in recombinant cell lines and its *in vitro* affinity at human 5-HT_7 receptors is similar to its affinity at human 5-HT_{1B} receptors (Bang-Andersen et al., 2011). Furthermore, systemic administration of the 5-HT_7 receptor antagonist SB269970 potentiates citalopram-induced increases in cortical extracellular 5-HT (Bonaventure et al., 2007). However, we find it unlikely that 5-HT_7 antagonism is related to region-specific effects on extracellular 5-HT levels in the brain regions studied here, as this receptor is expressed at similar levels in each region studied (vHC, NAc and mPFC; Neumaier et al., 2001). Furthermore, Lu AA21004 appears to have an approximately 10-fold weaker effect at rat 5-HT_7 receptors compared to human 5-HT_7 receptors in functional assays (Mørk et al., 2011), making this mechanism less plausible.

4.3. Lu AA21004 activates extracellular NE in mPFC and vHC while having only modest effects on LC neuron firing

Electrophysiological evidence suggests that increasing DRN activation (Segal, 1979) or local administration of an SSRI into the LC (Mateo et al., 2000) reduces firing rates at noradrenergic cell bodies, while 5,7-dihydroxytryptamine (5,7 DHT) lesions significantly increase LC firing rates (Haddjeri et al., 1997). Furthermore, 5-HT superfusion blocks potassium stimulated release of NE (Blandina et al., 1991; Matsumoto et al., 1995). Thus, 5-HT seems to play an inhibitory role in electrophysiological and neurochemical aspects of noradrenergic

neurotransmission. Interestingly, Lu AA21004 had opposing effects on these measures, including (1) a limited reduction in NE neuronal firing in the LC despite strong increases 5-HT efflux, and (2) increased NE release in the vHC and mPFC terminal regions. These effects were achieved despite a lack of adrenergic receptor or NE transporter affinity (Bang-Andersen et al., 2011), and were not mimicked by subchronic escitalopram at comparable SERT occupancy levels. These data suggest that Lu AA21004's 5-HT receptor effects modulate NE release.

Although general serotonergic activation inhibits NE release, microdialysis experiments consistently demonstrate that systemic application of 5-HT_{1A} receptor agonists elevate extracellular NE in the hypothalamus, hippocampus and cortex (Suzuki et al., 1995; Suwabe et al., 2000), and that these effects can be blocked by 5-HT_{1A} receptor antagonism (Suzuki et al., 1995). In the present study, Lu AA21004 produced a maximum 5-HT_{1A} receptor occupancy of about 44%; thus, it is plausible that 5-HT_{1A} agonism contributes to increased NE levels in the mPFC and vHC. The lack of an effect on NAc NE release can be explained by the lack of 5-HT_{1A} receptor expression in that region (Chalmers and Watson, 1991).

To our knowledge there are no published studies on the effects of activation or antagonism of the 5-HT_{1B} or 5-HT_7 receptors on NE release. However, 5-HT_{1B} receptors are known to exist as heteroreceptors in the LC on glutamatergic inputs originating from the paragigantocellularis nucleus as well as GABAergic inputs from the prepositus hypoglossi nucleus (Bobker and Williams, 1989). Thus, Lu AA21004's actions at 5-HT_{1B} receptors may have mixed electrophysiological effects on noradrenergic cell bodies, which may explain our observation that this compound had a limited effect on LC firing rates. Based on these data, it can be expected that any 5-HT_{1B}-mediated effects on NE release are due to activity in the terminal regions, rather than the cell body regions.

Currently available data on the role of 5-HT_3 receptors in NE release appears contradictory, with some researchers finding that 5-HT_3 receptor activation inhibits NE release and others finding that 5-HT_3 receptor activation potentiates NE release. This may be due to differences in the method used to elicit NE release. Hippocampal NE release induced by high potassium concentrations is blocked by 5-HT_3 receptor activation in vitro (Blandina et al., 1991) or *in vivo* (Matsumoto et al., 1995). Importantly, these attenuating effects are blocked by selective 5-HT_3 receptor antagonists such as ondansetron. However, the notion that 5-HT_3 receptors mediate an inhibitory effect on NE release is contradicted by studies that electrically stimulate NE release, which demonstrate that 5-HT_3 receptor activation potentiates NE release (Feurerstein and Hertting, 1986; Mongeau et al., 1994). Importantly, 5,7 DHT lesions have no effect on the ability of the 5-HT_3 receptor agonist 2-methyl-5-HT to potentiate electrically stimulated release (Mongeau et al., 1994), suggesting that 5-HT_3 receptors do not tonically regulate NE release. Given these conflicting data, it is unclear to what extent Lu AA21004's effects at 5-HT_3 receptors contribute to the observed NE increases, but evidence that 5-HT_3 receptors do not tonically regulate NE release makes it less likely that LuAA21004's potent antagonist effects at this receptor are relevant. This interpretation is in line with our data showing that NE release is only significantly increased at higher doses of Lu AA21004 (10 mg/kg), whereas 5-HT_3 occupancy reaches 94% occupancy at 0.1 mg/kg.

4.4. Lu AA21004 differentially modulates mesocortical and mesolimbic DA

Acute Lu AA21004 administration increased DA release in the vHC and mPFC, but not in the NAc. Furthermore, subchronic Lu AA21004 and escitalopram increased extracellular dopamine in the mPFC, but failed to do so in the vHC. Elevations in mPFC or vHC DA levels after Lu AA21004 or escitalopram may not be surprising given the consistency with which 5-HT elevating treatments also increase DA concentrations in these regions (Benloucif et al., 1993; Marcus et al., 2012; Smolders et al., 2008). But the lack of elevated DA outflow in the NAc is noteworthy for two reasons: (1) It suggests that Lu AA21004 differentially regulates the mesocortical and mesolimbic DA tracts originating from the VTA. (2) Given that 5-HT_{1B} activation is known to elevate NAc DA release (Benloucif et al., 1993; Boulenguez et al., 1996), the lack of increased NAc DA outflow provides indirect evidence that Lu AA21004 does not behave as a 5-HT_{1B} agonist *in vivo*. Furthermore, the observation that Lu AA21004 failed to alter DA firing rates in the VTA, even at high doses, suggests that Lu AA21004-mediated increases in extracellular DA are caused by effects at terminal regions, rather than DA cell bodies. This lack of an effect on VTA activity is surprising, given that SSRIs including escitalopram generally inhibit firing in this region (Di Mascio et al., 1998; Dremencov et al., 2009; Chernoloz et al., 2009), although there is some evidence that escitalopram can increase VTA cell firing (Schilström et al., 2011).

A limitation of the current study is that the pharmacological profile of Lu AA21004 is somewhat different in rodents versus humans (Bang-Andersen et al., 2011; Mørk et al., 2011). Lu AA21004 has about 10-fold lower in-vitro affinity for rat 5-HT_{1A} and 5-HT_7 receptors compared to human receptors, whereas it is more potent at the rat 5-HT_3 receptor. Thus, the results from the current study likely overestimate the effects at 5-HT_3 receptors and underestimate the effects at 5-HT_{1A} and 5-HT_7 receptors. Therefore, extrapolating results from rodents to humans should be done cautiously.

In conclusion, this study demonstrated that Lu AA21004 produces significant, dose-related elevations of 5-HT, NE, and DA in brain areas that are implicated in the pathogenesis of depression. Importantly, this compound's neurochemical effects were regionally specific, and were observed over a dose range that is active in behavioral models predictive of antidepressant and anxiolytic efficacy (e.g. Mørk et al., 2011). While further research is required to establish the full clinical implications of its pharmacological and neurochemical profile, Lu AA21004 appears to have properties that may make it an interesting therapeutic approach for depression and anxiety.

5. Disclosures

This research was supported by H Lundbeck A/S and the Takeda Pharmaceutical Company, Ltd.

Role of funding source

This study was funded by H Lundbeck A/S and the Takeda Pharmaceutical Company, Ltd. Employees of Lundbeck played a role in the design of experiments, as well as the collection, analysis

and interpretation of data. Lundbeck employees also played a role in the writing of and the decision to submit the present study.

Contributors

Alan Pehrson designed all receptor occupancy experiments, statistically analyzed microdialysis and *ex vivo* autoradiography data, and wrote the manuscript's first draft. Thomas Cremers and Maria van der Hart conducted all microdialysis experiments and participated in their design. Ceclie Betry collected and statistically analyzed data for the electrophysiology experiments, and participated in their design. Laerke Jorgensen conducted autoradiography experiments for SERT and 5-HT_{1B} receptor occupancy. Mathias Madsen conducted autoradiography experiments for SERT and 5-HT_3 receptor occupancy. Nasser Haddjeri participated in the design and analysis of all electrophysiology experiments. Bjarke Ebert participated in the design of all experiments. Connie Sanchez participated in the design of all experiments. All authors contributed to and have approved the final draft of this manuscript.

Conflict of interest

Alan Pehrson, Laerke Jørgensen, Mathias Madsen and Connie Sanchez are employees of Lundbeck Research USA, Inc. Bjarke Ebert is an employee of H. Lundbeck A/S. Nasser Haddjeri, INSERM employee, has received grants from Lundbeck and Solvay Pharmaceuticals. All other authors declare that they have no conflicts of interest

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