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Pharmacological and methodological aspects of the separation-induced vocalization test in guinea pig pups; a systematic review and meta-analysis

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ABSTRACT

The separation-induced vocalization test in guinea pig pups is one of many that has been used to screen for anxiolytic-like properties of drugs. The test is based on the cross-species phenomenon that infants emit distress calls when placed in social isolation.

Here we report a systematic review and meta-analysis of pharmacological intervention in the separation-induced vocalization test in guinea pig pups. Electronic databases were searched for original research articles, yielding 32 studies that met inclusion criteria. We extracted data on pharmacological intervention, animal and methodological characteristics, and study quality indicators.

Meta-analysis showed that the different drug classes in clinical use for the treatment of anxiety disorders, have comparable effects on vocalization behaviour, irrespective of their mechanism of action. Of the experimental drugs, nociception (NOP) receptor agonists proved very effective in this test. Analysis further indicated that the commonly used read-outs total number and total duration of vocalizations are equally valid. With regard to methodological characteristics, repeated testing of pups as well as selecting pups with moderate or high levels of vocalization were associated with larger treatment effects. Finally, reporting of study methodology, randomization and blinding was poor and Egger's test for small study effects showed that publication bias likely occurred.

This review illustrates the value of systematic reviews and meta-analyses in improving translational value and methodological aspects of animal models. It further shows the urgent need to implement existing publication guidelines to maximize the output and impact of experimental animal studies.

1. Introduction

In the search for novel pharmacological treatment of anxiety disorders, a wide range of animal models and tests has been used. Anxiety tests may either be performed in naïve animals or in animals in which aspects of the disease are modelled, *e.g.* by genetic modification or exposure to traumatizing stimuli. Furthermore, the read-outs quantified in the test itself may be based on innate anxiety, conditioned fear or conflict behaviour.

As of yet, it is unclear to what extent these approaches differ in predicting potential clinical efficacy and in detecting potential drugable targets. Together with the still limited understanding of the pathophysiology of anxiety disorders, this lack of knowledge may have hampered the translation of preclinical findings to the clinic (see Griebel and Holmes, 2013). In addition, limitations in study quality and design of experimental animal studies may also have contributed to this poor translation (Landis et al., 2012), notwithstanding issues concerning the outcome of clinical trials, such as high placebo responsiveness, patient selection and heterogeneity (Zimmerman et al., 2002).

As a first step to objectively characterize drug sensitivity and specificity of a particular anxiety test, we performed a systematic review and meta-analysis on pharmacological interventions in the separation-induced vocalization test in guinea pig pups and characterized methodological factors that may affect test outcome. In view of the concerns regarding study quality of experimental animal studies (Landis et al., 2012), we also determined prevalence of reporting of measures to reduce risk of bias and aimed to characterize if biases in publication are likely to occur.

The separation-induced vocalization test in guinea pig pups is based on the fact that pups emit distress calls when involuntarily separated from mother and littermates (Herman and Panksepp, 1978; Pettijohn, 1979a, 1979b). Narrative reviews indicate that, in contrast to other tests, the guinea pig pup vocalization test is sensitive to a broad range of antidepressants agents with anxiolytic properties

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(Sanchez, 2003; Borsini et al., 2002).This is an important observation in light of the highly valued predictive validity of animal models (Willner, 1984; Markou et al., 2009). Clinically, antidepressants are prescribed more frequently, and are preferred over benzodiazepines for the treatment of anxiety disorders (Baldwin et al., 2014; Chessick et al., 2006), although the evidence to support this has recently been questioned (Offidani et al., 2013; Rickels, 2013).

Besides detecting anxiolytic properties of antidepressant compounds, the guinea pig pup vocalization test has some additional valuable features. For certain neurotransmitter systems, human receptor pharmacology is more akin to that of guinea pigs than that of rats and mice, *e.g.* substance P (neurokinin $(NK)_1$ receptors (Rigby et al., 2005; Kramer et al., 1998; Leffler et al., 2009) and the serotoninergic system ($5\text{-}HT_{1B}$, $5\text{-}HT_{1D}$ receptors (Sipes and Geyer, 1996). In addition, brain development in guinea pig pups is in a more advanced state than that of rodents at birth (Clancy et al., 2007). Finally, the test induces relatively low discomfort to the animals, is easy to perform, and does not require special equipment, as guinea pig pups emit mainly audible calls upon separation (400–20.000 Hz; Berryman, 1976), which can easily be quantified. Together, these observations make this test interesting to further characterize pharmacologically and methodologically.

As outcome measures for our evaluation of drug efficacy in the separation-induced vocalization test in guinea pig pups, we used the total number and total duration of emitted vocalizations during social isolation. In the meta-analysis, we only included drugs that were hypothesized to reduce vocalization behaviour, whereas the systematic review was not confined to particular drug actions.

Literature suggests that both baseline levels of anxiety as well as sensitivity to the anxiolytic-like action of drugs in rats and mice are influenced by factors including strain (Griebel et al., 2000; Van Bogaert et al., 2006), early life stress (Paris and Frye, 2011; Schopper et al., 2011; Van Bogaert et al., 2006;Groenink et al., 2011) and environmental enrichment (Hendriksen et al., 2012). To determine if these factors affect drug efficacy in guinea pigs as well, we included these factors in our study. As behavioural studies indicate that age (Hennessy and Ritchey, 1987; Arch-Tirado et al., 2000), duration of social isolation (Monticelli et al., 2004), successive disturbances (Hennessy et al., 2006; Arch-Tirado et al., 2000), test environment (Hennessy and Ritchey, 1987) and presence of other guinea pigs in the test room (Pettijohn, 1979a), may influence vocalization behaviour in guinea pigs, we extracted information related to these factors as well. In addition, we studied if selection of pups based on pre-test vocalization levels, and repeated testing of animals impacted on study precision and effect size.

Finally, with regard to study quality, we assessed whether randomization, blinding and sample size calculations were reported and performed, since these factors are key in reducing the risk of biased results (Landis et al., 2012).

2. Material and methods

The systematic review was performed following a predefined protocol, the conditions of which are outlined below.

2.1. Literature search and study selection

Studies reporting drug treatment effects in separation-induced vocalization tests in guinea pigs were identified by electronic searching of Medline (via PubMed), Embase and Scopus, on January 14th, 2014. The search strategy was broad: it was designed to identify any study which measured vocalization behaviour in guinea pigs, it was not restricted by pharmacological intervention or language (for details see Table 1).

We applied the following criteria for in- and exclusion of peer-reviewed, original research articles:

a. Only studies using *guinea pigs* were included. All strains of guinea pigs were eligible, regardless of age and sex.
b. Only studies describing *effect of drug treatment* on *separation-induced* vocalizations compared with *control animals receiving vehicle treatmen*t were included.
c. Studies using a *systemic route of drug administration* were included. Studies using intra-cerebral or local infusion in specific brain areas were excluded.
d. Studies describing drug effects in animals that had received previous treatment, stress manipulations, lesions in the central nervous system or other treatments aimed at altering baseline levels of vocalizations were excluded.
e. Review articles not reporting original data were excluded.
f. If sufficient information to compute effect sizes could not be obtained from the article or from the primary or last author, studies were excluded from the meta-analysis.

To identify articles meeting the inclusion criteria (see Section 2.2), abstracts of retrieved articles were independently screened by two investigators (MV, LG), using EROS 3.0 (Early Review Organizing Software, Institute of Clinical Effectiveness and Health Policy, Buenos Aires, Argentina). Discrepancies were solved by discussion among the two investigators. In case information provided in the abstract was insufficient to decide upon in- or exclusion, both investigators screened the full article, following the same procedure.

Table 1
Search terms used to identify relevant articles.

Data base	Search string	Hits
PubMed	((“Guinea pigs”[MeSH Terms] OR (“guinea”[All Fields] AND “pigs”[All Fields]) OR “guinea pigs”[All Fields] OR (“guinea”[All Fields] AND “pig”[All Fields]) OR “guinea pig”[All Fields]) OR (“guinea pigs”[MeSH Terms] OR (“guinea”[All Fields] AND “pigs”[All Fields]) OR “guinea pigs”[All Fields] OR “cavia”[All Fields]) OR (“guinea pigs”[MeSH Terms] OR (“guinea”[All Fields] AND “pigs”[All Fields]) OR “guinea pigs”[All Fields] OR (“cavia”[All Fields] AND “porcellus”[All Fields]) OR “cavia porcellus”[All Fields]) OR (“guinea pigs”[MeSH Terms] OR (“guinea”[All Fields] AND “pigs”[All Fields]) OR “guinea pigs”[All Fields])) AND (Vocalization[All Fields] OR Vocalizations[All Fields] OR call[All Fields] OR calls[All Fields] OR (Distress[All Fields] AND vocalization [All Fields]) OR (Distress[All Fields] AND vocalizations[All Fields]))	201
Embase	(‘Guinea pig’/exp OR ‘guinea pig’ OR ‘cavia’/exp OR ‘cavia’ OR ‘*cavia porcellus*’/exp OR ‘*cavia porcellus*’ OR ‘guinea pigs’/exp OR ‘guinea pigs’) AND (‘vocalization’/exp OR ‘vocalization’ OR ‘vocalizations’ OR ‘distress vocalization’ OR ‘distress vocalizations’ OR ‘call’ OR ‘calls’ OR ‘vocalization’ OR ‘vocalizations’)	208
Scopus	(TITLE-ABS-KEY-AUTH(guinea pig) OR TITLE-ABS-KEY-AUTH(guinea pigs) OR TITLE-ABS-KEY-AUTH(*cavia porcellus*) OR TITLE-ABS-KEY-AUTH(cavia)) AND (TITLE-ABS-KEY-AUTH(Vocalization) OR TITLE-ABS-KEY-AUTH(Vocalizations) OR TITLE-ABS-KEY-AUTH(Distress vocalization) OR TITLE-ABS-KEY-AUTH (Distress vocalizations) OR TITLE-ABS-KEY-AUTH(Call) OR TITLE-ABS-KEY-AUTH(Calls) OR TITLE-ABS-KEY-AUTH(Vocalization) OR TITLE-ABS-KEY-AUTH (Vocalizations))	255

Table 2
Overview of pharmacological interventions in the isolation-induced vocalization test in guinea pig pups.

Paper	Drug	Route, ITI (min)	Dose (mg/kg)	Remark significance	IC50 (mg/kg)	*n*
GABA-ergic system						
Benzodiazepines						
Molewijk et al. (1996)	Alprazolam	*i.p.*, 30	0.3[a]/1[a]		0.32	8
Hodgson et al. (2007)	CDP	*i.p.*, 30	1/3/10[a]		NR	NR
Varty et al. (2005)	CDP	*i.p.*, 30	1/3/10[a]		NR	NR
Lamberty et al. (2004)	CDP	*i.p.*, 30	5/10[a]		NR	≥6
Rupniak et al. (2000)	CDP	*s.c.*, 30	0.3/3/10	*sign* NR	1,3	4–6
Kramer et al. (1998)	Diazepam	*s.c.*, 30	0,3/1/3	*sign* NR	NR	4–6
Molewijk et al. (1996)	Diazepam	*i.p.*, 30	1[a]/3[a]/10[a]		NR	8
Hudzik et al. (2003)	Diazepam	*s.c.*, 15	3/5[a]/10[a]		NR	4–8
Rupniak et al. (2000)	Diazepam	*i.p.*, 30	0.1/0.3/1/3	*sign* NR	0.7	4–6
Benzodiazepine receptor inverse agonists						
Molewijk et al. (1996)	DMCM	*i.p.*, 30	1/3/10	Inactive	NR	8
$GABA_A$ alpha$_1$ sub-unit receptor agonists						
Hudzik et al. (2003)	Zolpidem	*s.c.*, 15	1/3[a]/10[a]		NR	4–8
$GABA_A$ receptor agonists						
Molewijk et al. (1996)	Alcohol	*i.p.*, 30	100/300/1000[a]		NR	8
Serotonergic system						
Partial 5-HT_{1A} receptor agonists						
Molewijk et al. (1996)	BMY 7378	*i.p.*, 30	1/3/10	inactive	NR	8
Borowsky et al. (2002)	Buspirone	*i.p.*, 60	2[a]		NR	10
Kramer et al. (1998)	Buspirone	*s.c.*, 30	0,1/0,3/1/3	*sign* NR	NR	4–6
Molewijk et al. (1996)	Buspirone	*i.p.*, 30	0.3/1/3	Inactive	NR	8
Rupniak et al. (2000)	Buspirone	*s.c.*, 30	0.3/1/3	*sign* NR	0.45	4–6
Swanson et al. (2005)	Buspirone	*p.o.*, 120	2[a]		NR	10
5-HT_{1A} *receptor agonists*						
Molewijk et al. (1996)	8-OH-DPAT	*s.c.*, 30	0.1[a]/0.3[a]/1[a]		NR	8
Molewijk et al. (1996)	Flesinoxan	*i.p.*, 30	0.1/0.3[a]/1[a]		NR	8
5-HT_{1B} *receptor antagonists*						
Zhang et al. (2011)	AZD3783 μmol/kg	*s.c., NR*	0.2[a]/0.6[a]/2[a]		NR	12
Hudzik et al. (2003)	GR 127935	*s.c.*, 15	1/3/10[a]		NR	4–8
Dawson et al. (2006)	SB-616234-A	*i.p.*, 240	1/3[a]/10[a]		NR	7
Hudzik et al., 2003	ar-a000002	*s.c.*, 15	3/10/30[a]		NR	4–8
5-HT_{1D} *receptor agonists*						
Molewijk et al. (1996)	5-CT	*i.p.*, 30	0.3[a]/1[a]/3[a]		NR	8
5-HT_{2C} *receptor agonists*						
Molewijk et al. (1996)	m-CPP	*i.p.*, 30	1/3/10	Inactive	NR	8
5-HT_3 *receptor antagonists*						
Molewijk et al. (1996)	Ondansetron	*i.p.*, 30	0.001/0.01/0.1	Inactive	NR	NR
SSRIs						
Hudzik et al. (2003)	Citalopram	*s.c.*, 15	0.3/1/3	Inactive	NR	4–8
Molewijk et al. (1996)	Clomipramine	*i.p.*, 30	1/3/10[a]		NR	8
Dawson et al. (2006)	Fluoxetine	*i.p.*, 30	1/3[a]/10[a]		NR	6
Hudzik et al. (2003)	Fluoxetine	*s.c.*, 15	1/3/10	Inactive	NR	4–8
Rupniak et al. (2000)	Fluoxetine	*i.p.*, 30	1/3/10/30	*sign* NR	NR	4–6
Lamberty et al. (2004)	Fluoxetine	*i.p.*, 30	5/10[a]		NR	≥6
Rupniak et al. (2000)	Fluoxetine	*i.p.*, 30	1/3/10	*sign* NR	2.7	4–6
Steinberg et al. (2001)	Fluoxetine	*i.p.*, 30	1/3[a]/10[a]		NR	7–55
Chaki et al. (2005)	Fluvoxamine	*s.c.*, 30	NR[a]		NR	
Molewijk et al. (1996)	Fluvoxamine	*i.p.*, 30	3/10[a]/30[a]		NR	8
Yokoyama et al. (2009)	Fluvoxamine	*i.p.*, 30	30[a]			
Millan et al. (2010)	Paroxetine	*i.p.*, 30	0.16/2.5/10[a]		NR	8–19
TCAs						
Molewijk et al. (1996)	Desipramine	*i.p.*, 30	1/3/10[a]		NR	8
Yokoyama et al. (2009)	Desipramine	*i.p.*, 30	30[a]		NR	8–12
Kramer et al. (1998)	Imipramine	*s.c.*, 30	3/10/30	*sign* NR	NR	4–6
Lamberty et al. (2004)	Imipramine	*i.p.*, 30	16/32[a]		NR	≥6
Rupniak et al. (2000)	Imipramine	*s.c.*, 30	3/10/30	*sign* NR	5.4	4–6
Molewijk et al. (1996)	Maprotiline	*i.p.*, 30	1/3/10[a]		NR	8
SNRI's						
Rupniak et al. (2000)	Venlafaxine	*s.c.*, 30	3/10/30	*sign* NR	8.2	4–6
MAO-A inhibitors						
Molewijk et al. (1996)	Clorgyline	*i.p.*, 30	1/3/10[a]		NR	12
Kramer et al. (1998)	Phenelzine	*s.c.*, 30	3/10/30	*sign* NR	NR	4–6
Rupniak et al. (2000)	Phenelzine	*s.c.*, 30	3/10/30	*sign NR*	8.6	4–6

Table 2 (*continued*)

Paper	Drug	Route, ITI (min)	Dose (mg/kg)	Remark significance	IC50 (mg/kg)	*n*
Dopaminergic system						
Dopamine D_2 receptor antagonist						
Molewijk et al. (1996)	Haloperidol	*i.p.*, 30	0.1/0.3/1	Inactive	NR	NR
Psychostimulants						
Molewijk et al. (1996)	*d*-Amphetamine	*i.p.*, 30	0.3/1/3	Inactive	NR	NR
Substance P/Neurokinin receptor system						
NK_1 receptor antagonists						
Millan et al. (2010)	Aprepitant	*i.p.*, 30	0.63/10[a]/20[a]		NR	5–32
Kramer et al. (1998)	CGP49823	*s.c.*, 30	1/30	Inactive	NR	4–6
Rupniak et al. (2000)	CGP49823	*s.c.*, 30	1/30	Inactive	NR	4–6
Harrison et al. (2001)	Compound 3	*p.o.*, 240	0.1/0.3/1	*sign* NR	0.2	4–6
Brocco et al. (2008)	GR205,171	*i.p.*, 30	0.01/0.04[a]/0.16[a]/0.63		NR	7–20
Rupniak et al. (2000)	GR205,171	*s.c.*, 30	1/3/10	*sign* NR	2.7	4–6
Brocco et al. (2008)	GR226,206	*i.p.*, 30	0.04/0.16/0.63/2.5	Inactive	NR	5–24
Kramer et al. (1998)	L-733,060	*s.c.*, 30	1/3/10	*sign* NR	NR	4–6
Rupniak et al. (2000)	L-733,060	*s.c.*, 30	1/3/10	*sign* NR	3.2	4–6
Rupniak et al. (2000)	L-733,061	*s.c.*, 30	3/10/30	Inactive	NA	4–6
Kramer et al. (1998)	L-760,735	*s.c.*, 30	0.3/1/3	*sign* NR	NR	4–6
Rupniak et al. (2000)	L-796,325	*s.c.*, 30	3/10/30	Inactive	NA	4–6
Rupniak et al. (2000)	LY-303870	*s.c.*, 30	30	Inactive	NA	4–6
Steinberg et al. (2002)	SR240600	*i.p.*, 30	1/3[a]/10[a]		NR	7–55
Brocco et al. (2008)	Vestipitant	*i.p.*, 30	0.16/2.5[a]/10[a]		NR	7–16
Millan et al. (2010)	S41744	*i.p.*, 30	0.16[a]/0.63[a]/10[a]		NR	11–27
Substance P release inhibitor						
Hennessy et al. (2001)	(±) E-6006	*i.p.*, 30	3/10/30[a]/100[a]		NR	12
Hennessy et al. (2001)	(±) E-6006	*i.p.*, 30	30	Inactive	NR	16
Hennessy et al.,(2001)	(−) E-6006	*i.p.*, 30	30	Inactive	NR	16
Hennessy et al. (2001)	(+) E-6006	*i.p.*, 30	30[a]		NR	16
NK_2 receptor antagonists						
Steinberg et al. (2001)	SR 48965	*i.p.*, 30	3/10		NR	7–55
Steinberg et al. (2001)	SR 48968	*i.p.*, 30	0.3/1/3[a]/10[a]		NR	7–55
CRF systems						
CRF_1 receptor antagonists						
Hodgson et al. (2007)	CP154,526	*i.p.*, 30	3/10[a]/30[a]		NR	NR (df)
CRF receptor antagonist						
McInturf et al. (1996)	D-Phe-CRF	*s.c.*, 0	50[a]		NR	16
Hennessy et al. (1997)	D-Phe-CRF	*s.c.*, 0	50[a]		NR	12
Hennessy et al. (1997)	D-Phe-CRF	*s.c.*, 0	15/50/150	Inactive	NR	12
CRF receptor agonists						
Becker et al. (1993)	CRF	*s.c.*, 60	14 μg[a]		NR	7-8
Hennessy et al. (1991)	CRF	*s.c.*, 30	14 μg	Inactive	NR	12
Schiml-Webb et al. (2009)	CRF	*s.c.*, 0	10 μg[a]		NR	
Hennessy et al. (1995)	r/h CRF	*s.c.*, 30	7 μg[a]		NR	12
Hennessy et al. (1995)	r/h CRF	*s.c.*, 60	7 μg[a]/14 μg[a]		NR	12
Hennessy et al. (2011a)	r/h CRF	*s.c.*, 60	10 μg[a]		NR	11–12
Vasopressin system						
V_{1B} receptor antagonists						
Hodgson et al. (2007)	SSR149415	*i.p.*, 15	3/10/30[a]		NR	NR (df)
Central histaminergic system						
Histamine H_1 receptor antagonist						
Lamberty et al. (2004)	Chlorpheniramine	*i.p.*, 30	2/4/8/16	Inactive	NR	≥6
Lamberty et al. (2004)	Hydroxyzine	*i.p.*, 30	4.3/8.1/14.3[a]		NR	≥6
Histamine H_3 receptor agonist						
Yokoyama et al. (2009)	Immepip	*i.p.*, 15	3/10/30[a]		NR	8–12
Lamberty et al. (2004)	Immepip	*i.p.*, 30	5/10/20	Inactive	NR	≥6
Yokoyama et al. (2009)	*R*-α-methylhistamine	*i.p.*, 15	3/10[a]/30[a]		NR	8–12
Opioid system						
μ Opioid receptor agonists						
Herman et al. (1978)	Morphine	*s.c.*, 30	1[a]/2,5[a]/5[a]		NR	23
Herman et al. (1978)	Morphine	*s.c.*, 30	0.125[a]/0.25[a]/0.5[a]/0.75[a]		NR	20
Herman et al. (1978)	Morphine	*s.c.*, 30	1[a]/2.5[a]/5[a]		NR	10
μ Opioid receptor antagonists						
Herman et al. (1978)	Naloxone	*s.c.*, 30	1[a]		NR	9
Herman et al. (1978)	Naloxone	*s.c.*, 30	1	Inactive	NR	10
Immune system						
IL-10 receptor						
Hennessy et al. (2011b)	IL-10	*i.p.*, 90	1 μg	Inactive	NR	11–12

Table 2 (*continued*)

Paper	Drug	Route, ITI (min)	Dose (mg/kg)	Remark significance	IC50 (mg/kg)	*n*
CD4/TLR4/MD2 receptor complex						
Hennessy et al. (2011b)	LPS	*i.p.*, 90	0.075[a]		NR	12
Melanocortin receptor agonist						
Schiml-Webb et al. (2006)	α-MSH	*i.p.*, 60	25 μg	Inactive	NR	10
Schiml-Webb et al. (2009)	α-MSH	*i.p.*, 0	25 μg	Inactive	NR	10
Neuropeptidergic systems -miscellaneous						
BB_1/BB_2 receptor antagonist						
Merali et al. (2006)	PD 176252	*i.p.*, 30	1/3/10[a]/30[a]		NR	8
CCK_2 receptor agonist						
Molewijk et al. (1996)	CI-988	*i.p.*, 30	0.1/1/10	Inactive	NR	NR
GAL_3 receptor antagonist						
Swanson et al. (2005)	SNAP 37889	*p.o.*, 120	3[a]/10[a]/30[a]		NR	10
MCH_1 receptor antagonists						
Borowsky et al. (2002)	SNAP-7941	*i.p.*, 60	3/10[a]/30[a]		NR	10
Chaki et al. (2005)	ATC0175	*i.p.*, 60	1/3[a]/10[a]		NR	8
NOP receptor agonists						
Varty et al. (2005)	Ro64-6198	*i.p.*, 30	0.3/1[a]/3[a]		NR	NR (df)
Varty et al. (2008)	SCH 221510	*p.o.*, 120	0.3[a]/1[a]/3[a]		NR	10-11
Lu et al. (2011)	SCH 655842	*p.o.*, 120	0.3/1[a]/3[a]		NR	10

[a] Reported significant dose relative to vehicle; ITI injection test interval; IC50 half maximal inhibitory concentration; *n* number of animals per condition; 5-HT serotonin, BB bombesin; CDP chlordiazepoxide; CCK cholecystokinin; CRF corticotropin-releasing factor; GAL galanin; MAO mono-amine oxidase; MCH melanin-concentrating hormone; MSH melanocyte stimulating hormone; NOP nociception; SNRI serotonin-noradrenaline reuptake inhibitor; SSRI selective serotonin reuptake inhibitor; TCA tricyclic antidepressant; i.p. intraperitoneal; s.c. subcutaneous; p.o. oral; NR not reported; sign NR significance not reported; df group size estimated based on reported degrees of freedom. Gray line: insufficient data, experiment not included in meta-analysis.

2.2. Data extraction

2.2.1. Study characteristics

The following study characteristics were retrieved from the included articles by two independent investigators (MV, LG). Discrepancies were solved by discussion among the two investigators, or with a third investigator where necessary (BB).

2.2.1.1. Pharmacological intervention. Drug, mode of action of administered drug, dose and route of drug administration, injection test interval, vehicle control condition and treatment regimen (acute or chronic dosing) were extracted.

2.2.1.2. Animals. Strain, age, bodyweight, and sex of animals used, as well as origin of the pups (pregnant females from breeder, dam and pups from breeder, own breeding colony). Regarding housing conditions we also extracted data on housing (with mother, father, siblings), bedding (grid, sawdust, other), and enrichment (shelter yes/no, hay, extra vitamin C supplement).

2.2.1.3. Experimental conditions and study design. Test duration, detection method (hand count or automated call quantification), test box characteristics, time of day relative to dark–light cycle, presence of experimenter in test room, presence of other animals in test room. To determine to what extent study design and repeated use of animals may affect drug effects, we extracted information on total number of tests per pup (once/two or three times/more than three times), study design (full between/full within/repeatedly between/vehicle within), number of days between test days and statistical analysis used (parametric/non-parametric).

We initially aimed to extract data on the selection of pups by assessing whether any pre-screening was performed (yes/no). However, in an early phase of the extraction process we found that the method of selection differed substantially between studies. We therefore proceeded to record not only the presence of pre-screening, but also the selection criteria used. If no statement was made regarding selection of pups, this was interpreted as no selection.

2.2.2. Outcome measures

For outcome data extraction, articles were randomly divided between two investigators. Each investigator carried out initial data extraction from the allocated articles and the second investigator then checked all data entered by the first investigator (MV, BB).

We collected data for the total number of emitted vocalizations and the total duration of vocalizations. For each treatment and control group we extracted the number of animals, mean and variance (standard error of the mean or standard deviation). If outcome measures were presented graphically, data were measured using digital ruler software (Universal Desktop Ruler, AVPSoft.com). As median and interquartile range data cannot readily be transferred to a parametric scale, articles reporting such data could unfortunately not be included in the meta-analysis (Schiml-Webb et al., 2009, 2006; Hennessy et al., 2011b).

2.2.3. Study quality and risk of bias assessment

We assessed studies against the following quality indicators, adapted from SYRCLE's risk of bias tool (Hooijmans et al., 2014): reporting of randomization at any level (y/n), random allocation to intervention (y/unclear /n), reporting of blinding at any level (y/n), concealment of allocation (y/unclear/n), adequate blinding of outcome assessment (y/unclear/n), reporting of a sample size calculation (y/n), statement of a potential conflict of interest (y/n), and sponsorship (non-profit/(partly) profit/unknown). Articles were assessed independently by two investigators (MV, LG). Discrepancies were solved by discussion among the two investigators.

2.3. Meta-analysis

We included data from all articles that aimed to identify anxiolytic-like drug properties. In most articles, dose-response relationships were reported (see Table 2). However, since inclusion of drug effects obtained at suboptimal doses would interfere with interpretation of the effect size estimates, we only included data for the most effective dose tested (defined as the dose inducing the largest difference in

outcome measure relative to vehicle control condition) in the meta-analysis.

Data were analysed using Review Manager (version 5.2; Copenhagen, The Nordic Cochrane Centre, The Cochrane Collaboration). Data are presented as standardized difference in means and corresponding 95% confidence interval ((SMD [95% CI]): the mean of the experimental group minus mean of the control group, divided by the pooled SD of the two groups). The SMD was chosen because of expected differences in test duration, which may vary between 5 min up to 3 h. Since vocalizations decrease over time, it is not possible to average the number of calls *e.g.* per minute or per hour. Because of this, the unit of measurement for our outcome measures is unlikely to be uniform, and the SMD enables us to include different units of measurement in one analysis for each outcome measure. Although data were not independent for all studies, we treated them as such. To account for expected heterogeneity, we used the random effect model of DerSimonian and Laird. Subgroup analyses were performed to investigate the influence of study variables on treatment effect, as well as to explore possible causes for heterogeneity. Subgroups were omitted from the analysis if they contained less than three experiments, or were based on less than three articles. We pre-specified the following subgroup variables:

a. Drug class (to be defined separately for clinically active drugs and experimental drugs).
b. Strain (laboratory code for Dunkin Hartley/Hartley as reported in articles).
c. Gender (male/female/mixed).
d. Origin of the animals (colony/pregnant dam/dam with litter).
e. Age (young/mixed/old).
f. Test duration (5 min/10–15 min/ > 30 min).
g. Selection on pre-screening vocalization level (no selection/selection on moderate and high vocalization level/selection on high vocalization level).
h. Repeated testing (once/two or three times/more than three times).
i. Design (within design/between design).
j. Detection (by hand/automated).

After extracting data on the drugs tested in the included studies, we further categorized the clinically effective drugs into four categories: benzodiazapines, partial 5-HT_{1A} agonists, selective serotonin re-uptake inhibitors (SSRIs) and other anxiolytics/antidepressants. The experimental drugs were further categorized into μ-opioid receptor agonists, nociceptin (NOP) receptor agonists, neurokinin $(NK)_1$ receptor antagonists, substance P release inhibitors, 5-HT subtype receptor targeting drugs, and other neuropeptide and histamine targeting drugs.

Heterogeneity between studies was assessed using the I^2 and T^2 statistics. For the number of vocalizations, we corrected for multiple testing of five subgroup variables by adjusting the significance level to 0.0102 using the Holm–Bonferroni correction. Publication bias was investigated using funnel plotting and Egger regression.

3. Results

3.1. Study selection and search results

We identified 273 unique publications from the electronic search, of which 241 publications were excluded (see Fig. 1). This review is therefore based on data from 32 articles. We contacted first and/or last authors in an attempt to obtain missing data or study characteristics. We received a response from five authors, two of which were able to supply additional data.

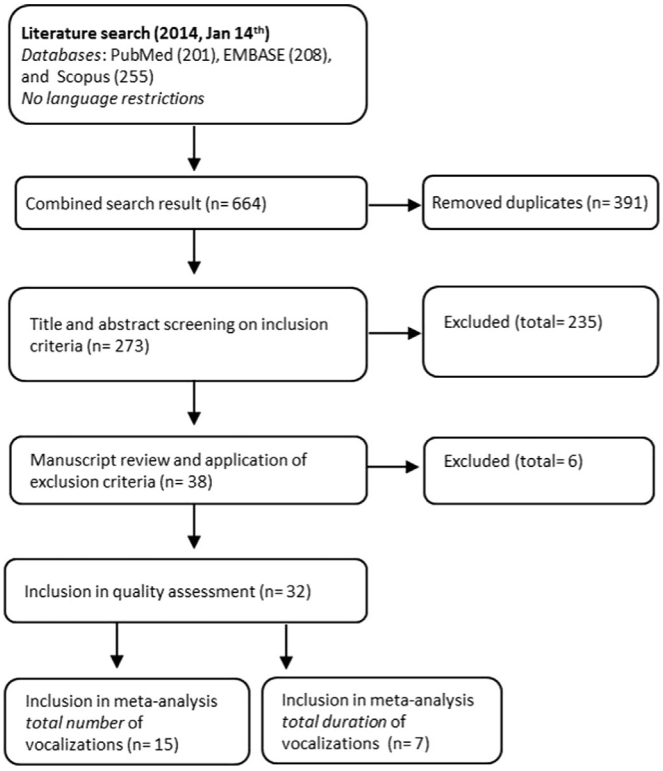

Fig. 1. Flow chart of article selection process.

3.2. Description of included studies

3.2.1. Pharmacological interventions

In Table 2, drug effects as reported in the included articles are summarized. Drugs are sorted by neurotransmitter system and mechanism of action. In total, 105 experiments were reported, involving either registered anxiolytics/antidepressants with anxiolytic properties (37 experiments) or experimental compounds (68 experiments). In general, findings were consistent between studies that tested the same compound. With the exception of the NK_1 receptor antagonist GR205,171, the reported effective dose-ranges were comparable.

Effects of clinically active drugs were reported in fourteen of the 32 articles, five of which tested more than one registered anxiolytic/antidepressant. In total, sixteen different registered anxiolytic/antidepressant were tested. SSRIs (ten experiments) and benzodiazepines (nine experiments) were tested most often. Paroxetine (six experiments), chlordiazepoxide (four experiments) and diazepam (four experiments) were most frequently used as representatives of these drug classes. Partial 5-HT_{1A} receptor agonists were tested six times. In twelve of the fourteen articles, all reference compounds significantly reduced vocalization behaviour. One study however, reported the absence of effect of partial 5-HT_{1A} receptor agonists, whereas a wide range of other clinically active drugs did reduce total number of vocalizations in that study (Molewijk et al., 1996). Hudzik and co-workers reported that the SSRIs citalopram and fluoxetine had no effect in the vocalization test, and that high doses of the benzodiazepine diazepam were needed to reduce vocalization behaviour (Hudzik et al., 2003).

With regard to experimental drugs, 54 different compounds were tested in 68 experiments. As shown in Table 2, a variety of neurotransmitter systems was targeted. In total, the effects of 29 different mechanisms of action were investigated. Compounds targeting the substance P/neurokinin system or the serotonin system were tested most frequently.

In 79 of the 105 experiments, the compound under study reduced the number and/or the total duration of vocalization,

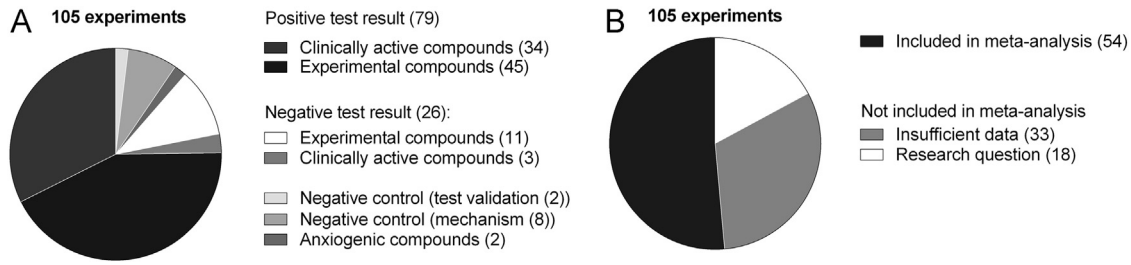

Fig. 2. Overview of experiments included in (A) the systematic review, and in (B) the meta-analysis.

which is indicative of an anxiolytic effect (Fig. 2A). In 26 experiments the compound under study was inactive (including the three registered anxiolytics/antidepressants mentioned above). In most cases, this absence of a drug effect was anticipated by the authors, as shown in Fig. 2A. These compounds were tested to show *e.g.* stereo-selective activity (NK_1 and NK_2 receptor antagonists (Kramer et al., 1998; Steinberg et al., 2001; Rupniak et al., 2000), to determine selectivity of the guinea pig pup vocalization test (the psychostimulant *d*-amphetamine and the antipsychotic haloperidol (Molewijk et al., 1996), or to study the role of the immune system in passive behaviour, which is induced upon prolonged social isolation (*e.g.* α-MSH, LPS and IL-10; Schiml-Webb et al., 2006, 2009; Hennessy et al., 2011b).

In addition, the two anxiogenic compounds studied in this test, the inverse benzodiazepine receptor agonist DMCM and the 5-HT_{2C} receptor agonist m-CPP were reported to be inactive (Molewijk et al., 1996), as were the 5-HT_3 receptor antagonist ondansetron, and the cholecystokinin $(CCK)_2$ receptor agonist CI-988 (Molewijk et al., 1996).

3.2.2. Animal characteristics

No article specified the laboratory code of the strain under study. Instead, articles referred to the guinea pig strains using generic names. The use of Dunkin–Hartley was reported in twelve articles, whereas eight articles used Hartley guinea pigs. Twelve articles did not report which guinea pig strain was used (see Table 3).

For most studies (twelve articles), pups were obtained by ordering pregnant dams. If specified, these dams were around six weeks pregnant (see Table 3; guinea pig gestation period is 60–73 days). Alternatively, dams with litter (1–3 days old) were purchased from suppliers (six articles), or pups were bred in a colony kept within the own laboratory (ten articles). In four articles, origin of the pups was not specified.

The age at which pups were tested varied from postnatal day (PND) 4 to PND 42, and was often related to the study design. Generally, when pups were tested once (between subjects design), pups were not older than approximately 3 weeks, and the age-range was limited to one week. When pups were tested repeatedly, or in a full within subjects-design (receiving all doses and vehicle), they were up to 4 to 5 weeks old, and age within a study ranged from two to four weeks (see Table 3 for details). Body weight was only reported once (not shown).

As shown in Table 3, fourteen out of 32 articles stated that both male and female pups were tested. Three of these articles actively controlled for sex differences, either by allocating equal numbers of both sexes to experimental groups (Hennessy et al., 1995, 2001), or by including sex as a factor in the statistical design (Hennessy et al., 1997).

Pups were always housed with mother and siblings, but the number of nests and siblings within a cage varied, and was often not well specified. Cages, bedding material, and presence of cage enrichment were also poorly reported. Therefore, it was not possible to categorize these variables in a meaningful way (data not shown).

3.2.3. Experimental characteristics and study design

The guinea pig infant separation calls have been classified as "whistles", which are mainly audible sounds (Berryman, 1976). The first studies indeed specified the type of calls quantified in the guinea pig pup separation test, as high pitched calls (*e.g.* whistles). This information however, is lacking from most articles included in this review.

Only two articles, both using automated quantification of calls, reported minimal length of a vocalization and minimal interval between vocalization to qualify as two separate calls (Brocco et al., 2008; Millan et al., 2010), whereas another paper used sonographic recordings to identify calls with specified characteristics (Molewijk et al., 1996). Quantification by hand was most frequently used (21 articles); automated quantification was used in three articles.

Test duration of the guinea pig pup vocalization test is typically 5 min (fifteen articles), although 2 min test (one article) and test durations from 12 to 15 min have also been performed (five articles, Table 3). Articles using a considerable longer test duration (30 min or more, ten articles), studied mechanisms underlying the shift from active to passive coping behaviour during prolonged social isolation.

In eighteen out of the 32 included articles, pups were selected based on their vocalization behaviour during a pre-test. Selection criteria used, ranged from 30 s to 200 s within 5 min, and from 200 to 600 calls within 5 min (see Table 3).

Depending on the study design (between subjects or within-subjects study design), pups were tested once (nine articles), two or three times (nine articles), or more frequently (eight articles; see Table 4). Five articles did not report test frequency. In some within-subjects studies, each pup received each dose and vehicle treatment (nine articles), in four articles pups received one dose and vehicle, in another four articles pups received two or three conditions out of four or five possible conditions, including vehicle. Nine articles used a between subjects design, with pups being tested only once. Six articles did not (clearly) report the study design or repeated use of pups.

We aimed to extract data on test variables including test box (illumination), time of day relative to dark–light cycle, relocation of pups or whole nest from facility to test room, presence of experimenter in test room, and presence of other animals in test room.

It proved difficult however, to extract this information from most articles (except for time of day relative to dark–light cycle). Information was either missing (*e.g.* presence of other animals in test room not reported in 50% of the articles), or reported in general terms (*e.g.* 'dimly lit'). It was therefore not possible to structure these variables in a meaningful way.

3.3. Risk of bias and quality of reporting

As shown in Table 4, only three out of 32 included articles (9.5%) reported that the study was randomized, without further

Table 3
Animal characteristics and test characteristics of the separation-induced vocalization test in guinea pig pups.

Article	Strain	Origin animals	Age (days)	Sex	Pre-screening (selection criterion)	Test duration (min)	Outcome measure	Included in MA exclusion/remark
Becker and Hennessy (1993)	Hartley	own colony	20-21	M/f (=)	NR [no]	30	Number	N, different aim
Borowsky et al. (2002)	NR	NR	14	NR	Yes (NR)	5	Number	Y
Brocco et al. (2008)	NR	Pregnant dam (6 wk)	7–31	NR	> 30 s within 5 min	5	Number, duration	Y
Chaki et al. (2005)	NR	Pregnant dam	7–28	NR	> 200 s within 5 min	5	Number, duration	Y
Dawson et al. (2006)	Dunkin Hartley	Pregnant dam	8–27	NR	> 100 s within 15 min	15	−, Duration	Y
Harrison et al. (2001)	NR	NR	NR	NR	NR [no]	NR	% Inhibition (NR)	N, insufficient data
Hennessy et al. (1991)	Hartley	Own colony	20–26	M/f (=)	NR [no]	30	Number	N, different aim
Hennessy et al. (1995)	Hartley	own colony	20–21	m/f (=)	NR [no]	30	Number	N, passive behaviour
Hennessy et al. (1997)	Dunkin Hartley	Own colony	20–26	M/f (=)	NR [no]	60	Number	N, anxiogenic properties
Hennessy et al. (2001)	Hartley	Own colony	13–15	M/f (=)	NR [no]	30	Number	Y
Hennessy et al. (2011a)	NR	Own colony	21–23	M/f (=)	No	180	Number	N, passive behaviour
Hennessy et al. (2011b)	NR	Own colony	18–25	M/f (=)	NR [no]	60	Number	N, medians, passive behaviour
Herman et al. (1978)	Dunkin Hartley	Pregnant dam	7–17	M/f	Yes (NR)	15	Number	Y
Hodgson et al. (2007)	Dunkin Hartley	Pregnant dam	5–21	M/f	NR [no]	5	Number	Y (df)
Hudzik et al. (2003)	Dunkin Hartley	Pregnant dam (4 wk)	5–35	NR	Yes (NR)	2	Number	N, insufficient data
Kramer et al. (1998)	NR	NR	NR	NR	> 300 s within 15 min	15	% Inhibition duration	N, insufficient data
Lamberty et al. (2004)	Dunkin Hartley	Dam+litter	14	NR	> 300 s within 12 min	12	−, Duration	Y
Lu et al. (2011)	Dunkin Hartley	Dam +litter	NR	NR	# > 200 within 5 min	5	Number	Y
McInturf and Hennessy (1996)	Hartley	Own colony	20–26	M/f (=)	No	30	Number	N, passive behaviour
Merali et al. (2006)	Dunkin Hartley	Dam +litter	NR	NR	# > 500 within 5 min	5	Number	Y
Millan et al. (2010)	Dunkin Hartley	Pregnant dam (6 wk)	6–27	NR	> 30 s within 5 min	5	Number, duration	Y
Molewijk et al. (1996)	NR	Pregnant dam	6–23	NR	# > 200 within 5 min	5	Number	Y
Rupniak et al. (2000)	NR	Pregnant dam	14–42	NR	> 300 s within 15 min	15	% Inhibition (NR)	N, insufficient data
Schiml-Webb et al. (2006)	NR	Own colony	19–26	M/f (=)	NR [no]	180	Number	N, passive behaviour
Schiml-Webb et al. (2009)	NR	Own colony	21–26	M/f (=)	NR [no]	60	Number	N, medians; anxiogenic
Steinberg et al. (2001)	Dunkin Hartley	Dam+litter	12–24	NR	> 120 s within 5 min	5	−, Duration	Y
Steinberg et al. (2002)	Hartley	NR	9–23	NR	> 120 s within 5 min	5	−, Duration	Y
Swanson et al. (2005)	Hartley	Pregnant dam (6 wk)	14	NR	NR [no]	5	Number	Y
Varty et al. (2005)	Hartley	Dam+litter	6–20	M/f	# > 200 within 5 min	5	Number	Y (df)
Varty et al. (2008)	Dunkin Hartley	Dam+litter	6–20	M/f	# > 200 within 5 min	5	Number	Y
Yokoyama et al. (2009)	NR	Pregnant dam	7–8	NR	No. of > 600 within 5 min	5	Number	Y
Zhang et al. (2011)	Dunkin Hartley	Pregnant dam	4–25	NR	Yes (NR)	5	Number	Y

NR=not reported; dam with litter ordered from breeder/pregnant dam from breeder; selection not reported, interpreted as NO selection; # number of calls; MA meta-analysis; m/f both male and female pups ((=) reported absence of sex difference); # total number of calls; duration total duration of calls; df: group size (n) estimated using degrees of freedom; gray not included in meta-analysis. Insufficient data *e.g.* data not reported/shown, number of animals tested was not reported, distortion not reported, overlapping error bars, no data for vehicle condition. Different aim not studying anxiolytic drug properties.

specification of how the allocation sequence was generated and applied. Blinding was reported in eight articles (25%), which was further specified as blinding of observer in five of these articles, and absence of blinding of observer in two articles. Only two articles addressed incomplete outcome data, whereas no article reported a sample size calculation. Five articles included a conflict of interest statement, two of which reported a potential conflict of interest. Consequently, all articles are considered to be of poor quality.

3.4. Meta-analysis of outcome measures

Meta-analysis was performed on nineteen articles, reporting on 55 experimental comparisons. In the other thirteen articles (50 experiments), required data were missing (gray lines in Table 2, Fig. 2B), or no anxiolytic-like drug properties were studied (see Table 3, Fig. 2B).

Fifteen articles reported drug effects on the total number of vocalizations, which included 43 vehicle-drug comparisons, using

Table 4
Study characteristics and risk of bias assessment scores.

	Study characteristics			Study quality			
Article	**Quantification method**	**Number of tests per pup**	**Design**	**Randomization reported**	**Blinding reported**	**Sample size calculation**	**Conflict of interest statement**
Becker et al. (1993)	Hand	1	Full between	NR	NR	NR	NR
Borowsky et al. (2002)	NR	2	Repeatedly, between	NR	NR	NR	Y
Brocco et al. (2008)	Automated	5	Full within	Y	NR	NR	Y (no conflict)
Chaki et al. (2005)	Recorded	5	Full within	NR	NR	NR	NR
Dawson et al. (2006)	Automated	4	Full within	Y	Y	NR	NR
Harrison et al. (2001)	NR	NR	NR	NR	NR	NR	NR
Hennessy et al. (1991)	Hand	1	Full between	NR	NR	NR	NR
Hennessy et al. (1995)	Hand	1	Full between	NR	NR	NR	NR
Hennessy et al. (1997)	Hand	2	Vehicle within	NR	NR	NR	NR
Hennessy et al. (2001)	Hand	1	Full between	NR	NR	NR	NR
Hennessy et al. (2011a)	Hand	1	Full between	NR	Y (not blinded)	NR	Y (no conflict)
Hennessy et al. (2011b)	Hand	1	Full between	NR	NR	NR	NR
Herman and Panksepp (1978)	Hand	5	Full within	NR	NR	NR	NR
Hodgson et al. (2007)	Hand	NR	NR	NR	Y	NR	NR
Hudzik et al. (2003)	Hand	4	Full within	NR	NR	NR	NR
Kramer et al. (1998)	NR	NR	NR	NR	NR	NR	NR
Lamberty et al. (2004)	Hand	1	Full between	Y	NR	NR	NR
Lu et al. (2011)	Hand	NR	NR	NR	Y	NR	NR
McInturf and Hennessy (1996)	Hand	2	Full within	NR	NR	NR	NR
Merali et al. (2006)	Recorded	1	Full between	NR	NR	NR	NR
Millan et al. (2010)	Automated	5	Full within	NR	NR	NR	Y
Molewijk et al. (1996)	Recorded	5	Full within	NR	NR	NR	NR
Rupniak et al.,(2000)	NR	6	Within	NR	NR	NR	NR
Schiml-Webb et al. (2006)	Hand	3	Vehicle within	NR	Y (not blinded)	NR	NR
Schiml-Webb et al. (2009)	Hand	2	Full within	NR	NR	NR	NR
Steinberg et al. (2001)	Hand	3	Vehicle within	NR	NR	NR	NR
Steinberg et al. (2002)	Hand	3	Vehicle within	NR	NR	NR	NR
Swanson et al. (2005)	NR	2	Repeatedly, between	NR	Y	NR	Y (no conflict)
Varty et al. (2005)	Hand	6	Between	NR	Y	NR	NR
Varty et al. (2008)	Hand	3	Repeatedly, between	NR	NR	NR	NR
Yokoyama et al. (2009)	Hand	1	Full between	NR	Y	NR	NR
Zhang et al. (2011)	Hand	NR	NR	NR	NR	NR	NR

NR not reported; Y yes; gray not included in meta-analysis.

977 guinea pig pups in total. Seven articles reported drug effects on the total duration of vocalizations. These included twenty comparisons, using 762 pups. Drug effects on both the total number and total duration of calls were analysed for eight experiments from three articles (Brocco et al., 2008; Chaki et al., 2005; Millan et al., 2010).

3.4.1. *Pharmacological interventions*

3.4.1.1. Total number of vocalizations. Results for drug effects on total number of calls are summarized in Fig. 3. When pooling data from all included experiments, drug treatment was favoured over vehicle (SMD −1.99[−2.33, −1.64], 15 articles, 43 experiments, 977 animals). Absolute and between study heterogeneity for all studies combined were reasonably high (T^2=0.97; I^2=77%).

Subgroup analysis showed that all drug classes (benzodiazepines, partial 5-HT_{1A} receptor agonists, SSRIs, μ opioid receptor agonists, NOP receptor agonists, NK_1 receptor antagonists, and substance P inhibitors) reduced the total number of vocalizations. The anxiolytic effects of the included drug classes were well within in the same range (see Table 5, Fig. 3).

For a number of drug classes, data were insufficient to create subgroups based on their mechanism of action (Table 5). For visualization in the forest plot, these remaining compounds were therefore grouped (but not pooled), based on their clinical effect (anxiolytics/antidepressants miscellaneous) or neurotransmitter system targeted (5-HT subtype receptor miscellaneous, neuropeptides and histamine miscellaneous, experimental miscellaneous).

A subgroup analysis comparing three classes of clinically active anxiolytics (benzodiazepines, partial 5-HT_{1A} receptor agonists and SSRIs), showed that the effect of these drug classes on total number of vocalizations did not differ (Fig. 3, Table 5). Overall and between study heterogeneity in the SSRI subgroup was low (I^2= 0%, T^2=0.0), compared to the other two classes (I^2= 56%, T^2=0.48 and I^2= 75%, T^2=0.96 for benzodiazepines and partial 5-HT_{1A} receptor agonists, respectively).

Out of all experimental drug classes studied, NOP receptor agonists appeared to have the largest effect (SMD −4.39 [−5.41, −3.37], three articles, three experiments, 60 animals). Heterogeneity was low in this subgroup (τ^2=0 and I^2 0%), and the reduction in vocalizations after treatment with NOP receptor agonists was large, when compared to benzodiazepines, partial 5-HT_{1A} receptor

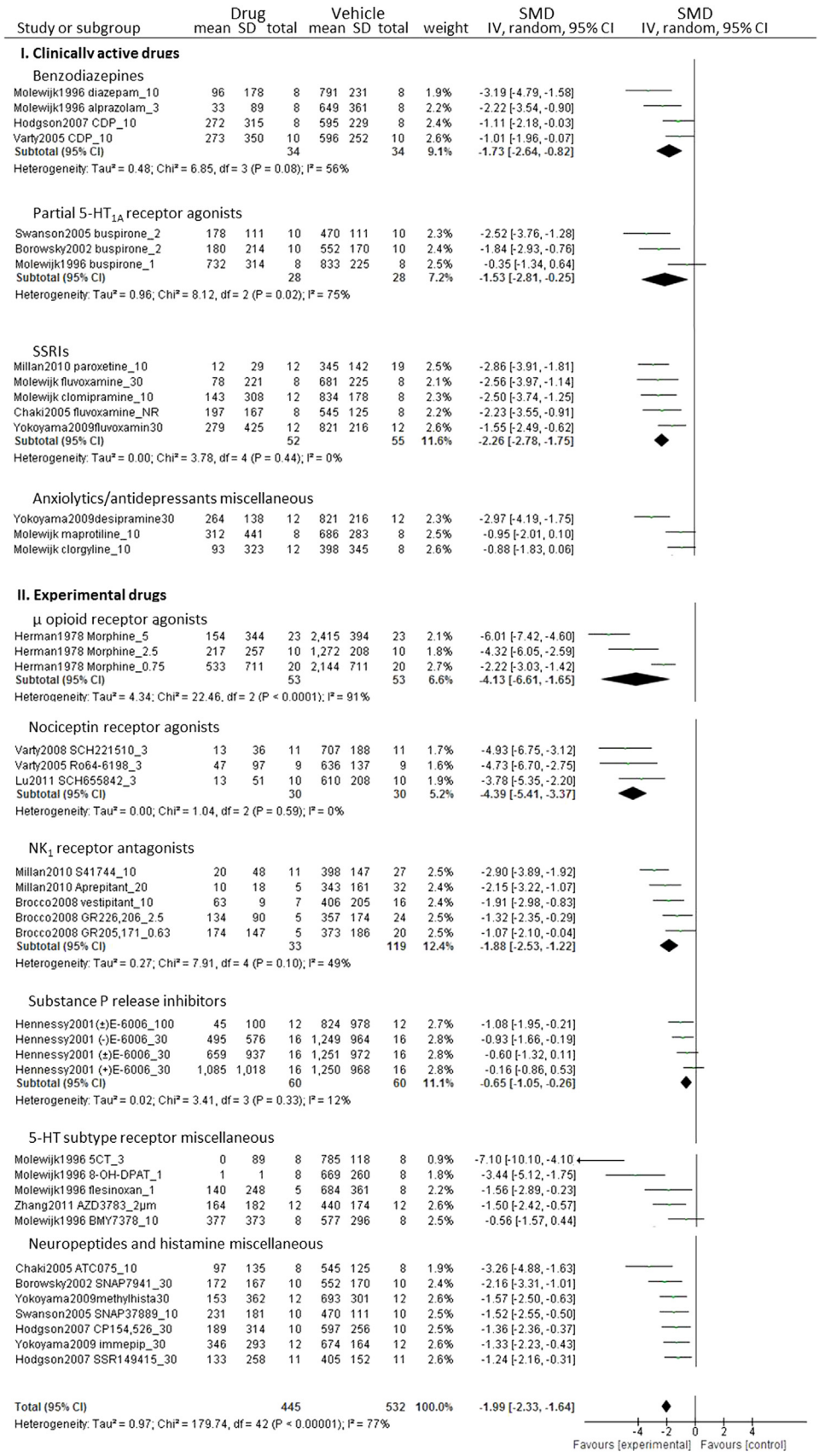

Fig. 3. Forest plot of all included studies on the effect of pharmacological intervention on total number of calls, sorted by subgroups and divided into I. clinically active and II. experimental drug categories (upper and lower panel). The forest plot displays the SMD, 95% CI and relative weight of the individual studies. Experiments are labelled by author, publication year, compound and dose included in the meta-analysis.

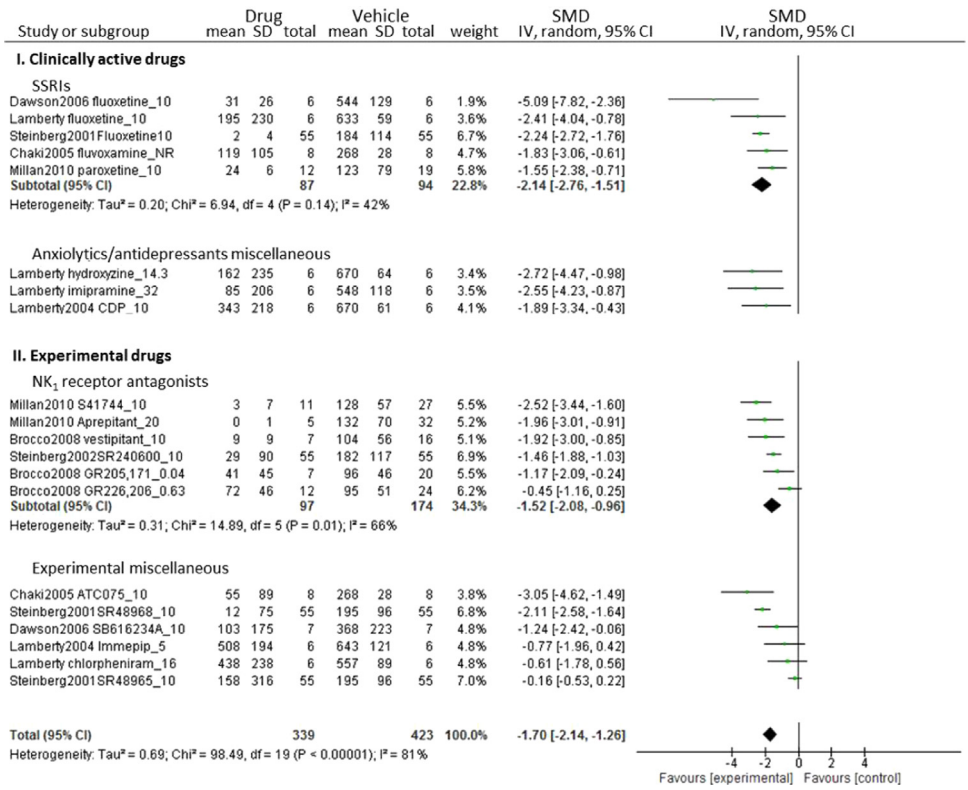

Fig. 4. Forest plot of all included studies on the effect of pharmacological intervention on total duration of calls. Drugs are sorted by subgroups and divided into I. clinically active and II. experimental drug categories (upper and lower panel). The forest plot displays the SMD, 95% CI and relative weight of the individual studies. Studies are labelled by author, publication year, compound and dose included in meta-analysis.

agonists and SSRIs (ΔSMD respectively 2.66[1.29, 4.03], 2.86[1.22, 4.50] and 2.13 [0.99, 3.27]).

3.4.1.2. Total duration of vocalizations. Pooling of all available experiments showed that pharmacological intervention reduced the total duration of vocalizations relative to vehicle treatment (SMD −1.70, [−2.14, −1.26], 20 experiments, 762 animals). Total and between study heterogeneity were reasonably high (I^2=81%; T^2=0.69; Fig. 4).

Subgroup analysis for duration of vocalizations were performed for SSRIs and NK_1 receptor antagonists. For a number of other drug classes, data were insufficient to create subgroups based on their mechanism of action (Table 6). For visualization in the forest plot, these remaining compounds were therefore grouped (but not pooled) in two categories (anxiolytics/antidepressants miscellaneous and experimental miscellaneous).

Total duration of vocalizations was reduced for both SSRIs and NK_1 receptor antagonists (Fig. 4), and effect sizes did not differ between these subgroups (ΔSMD not significant).

Heterogeneity was modest in both groups (I^2=42% and 66%, and T^2=0.20 and 0.31, respectively).

3.4.2. Meta-analysis of methodological characteristics

3.4.2.1. Total number of vocalizations. Since only generic names were used to indicate the guinea pig strain studied, stain differences were not analyzed.

Subgroup analysis indicated an overall effect of the variable origin of pups on treatment efficacy (see Table 5). Between-subgroup analysis, could only be performed for the subgroups pregnant dam and dam with litter. In both these subgroups, pharmacological treatment was favoured over vehicle and comparable effect sizes were obtained.

To determine the impact of age on drug effects on vocalization behaviour, experiments were categorized in three subgroups: young (PND 7–PND 17), mixed age (PND 4–PND 31) and old (PND18–26; insufficient data). Subgroup analysis showed an overall effect of the variable age on treatment efficacy. However, no difference between the young and the mixed age groups was observed (Table 5). For the variable test duration, subgroups were too small to perform reliable analysis.

To study the effect of animal selection on effect size and precision of test results, experiments were categorized in three subgroups: no selection, selection on moderate to high vocalization level (total duration at least 30 s within 5 min, or at least 200 calls within 5 min), and selection on high vocalization level (at least 120 s within 5 min, or at least 500 calls within 5 min). In all subgroups, pharmcological treatment was favoured over vehicle (Table 5). Subgroup analysis showed an overall effect of the variable selection on treatment efficacy. Comparisons between subgroups revealed that treatment effect in experiments without selection was significantly smaller compared to the moderate/high (ΔSMD 1.15[0.48, 1.82] and high subgroups (ΔSMD 0.78[0.10, 1.46]. The effect of pharmacological treatment did not differ between the moderate/high and high subgroups (ΔSMD 0.37[−0.40, 1.14]).

To determine the effect of repeated testing on treatment effects, we compared three subgroups, based on the total number of tests a pup was exposed to: one test, two or three tests or more than three tests. Overall, the variable repeated testing had a significant effect on treatment efficacy. Pharmacological treatment reduced the total number of calls in all subgroups (Table 5). Between-subgroup comparisons indicated that the effect of treatment was significantly smaller in pups tested once (SMD −1.16 [−1.61, −0.70]) than in pups tested two to three times

Table 5
Subgroup statistics for total number of vocalizations.

Subgroup	# Articles	# Experiments	# Animals	SMD[CI]
All studies	**15**	**43**	**977**	**−1.99 [−2.33, −1.64]**
Drug class				
Registered	10	13	247	−1.80 [−2.29, −1.30]
Test for subgroup differences NS				
Benzodiazepines	3	4	68	−1.73 [−2.64, −0.82]
5-HT_{1A} partial agonists	3	4	72	−1.53 [−2.81, −0.25]
SSRIs	4	5	107	−2.26 [−2.78, −1.75]
Anxiolytics/antidepressants miscellaneous	2	3	60	Not pooled
Experimental				
Test for subgroup differences NA				
μ Opioid receptor agonists	1	3	106	−4.13 [−6.61, −1.65]
NOP receptor agonist	3	3	60	−4.39 [−5.41, −3.37]
NK_1 receptor antagonists	2	5	152	−1.88 [−2.53, −1.22]
Substance P release inhibitors	1	4	120	−0.65 [−1.05, −0.26]
5-HT subtype receptor miscellaneous	2	4	69	Not pooled
Neuropeptides and histamine miscellaneous	6	8	163	Not pooled
Origin				
Test for subgroup differences $P < 0.00001$				
Colony	1	4	120	−0.65 [−1.05, −0.26]
Pregnant dam	9	32	720	−2.06 [−2.44, −1.69]
Dam with litter	4	5	97	−2.95 [−4.61, −1.29]
Not reported	1	2	40	−1.99 [−2.78, −1.20]
Age				
Test for subgroup differences $P < 0.00001$				
Young < 17 days	5	15	402	−1.93 [−2.55, −1.32]
Mixed (4–31 days)	8	26	538	−2.00 [−2.43, −1.58]
Old > 18–26 days	0	0	0	NA
Not reported	2	2	37	−2.34 [−5.02, 0.34]
Test duration				
Test for subgroup differences NA				
5 min	13	36	751	−1.96 [−2.28, −1.64]
10–15 min	1	3	106	−4.13 [−6.61, −1.65]
> 30 min	1	4	120	−0.65 [−1.05, −0.26]
Selection				
Test for subgroup differences $P < 0.00001$				
None	3	9	218	−1.06 [−1.45, −0.66]
Moderate/high vocalization	6	21	444	−2.21 [−2.76, −1.67]
High vocalization	3	7	145	−1.84 [−2.39, −1.29]
Not reported	3	6	170	−2.91 [−4.11, −1.70]
Repeated testing				
Test for subgroup differences $P < 0.00001$				
Once	3	9	233	−1.16 [−1.61, −0.70]
2-3 times	3	5	102	−2.41 [−3.32, −1.50]
4 or more times	6	24	540	−2.35 [−2.88, −1.81]
Not reported	3	5	102	−1.63 [−2.34, −0.92]

NA not applicable; NS not significant; # number; SMD standardized mean difference; CI confidence interval; Subgroups consisting of less than three experiments and/or less than three articles were excluded from between subgroup analyses.

(SMD −2.41 [−3.32, −1.50]; ΔSMD 1.25 [0.23, 2.27]) and pups tested more than three times (SMD −2.35 [−2.88, −1.81], ΔSMD 1.19 [0.48, 1.89]).

3.4.2.2. Total duration of vocalizations. No subgroup analyses were performed for total duration of vocalizations, because of insufficient numbers of experiments and/or articles, except for the variable selection of pups (see Table 6). The effect of pharmacological treatment did not differ between the moderate/high and high selection subgroups (SMD not significant). There were insufficient data to compare these subgroups with the no-selection subgroup.

3.4.2.3. Comparison of outcome measures. When pooling data from all nineteen included articles, the two outcome measures did not differ in the effects induced by the pharmacological interventions (total number of calls SMD −1.99, [−2.33, −1.64], 43 experiments, 977 animals (Fig. 3) *versus* total duration SMD −1.70, 95% CI [−2.14, −1.26], 20 experiments, 762 animals (Fig. 4). Distortion (CI), absolute heterogeneity ($\tau^2 = 0.97$ and 0.69, respectively), and heterogeneity between studies ($I^2 = 77\%$ and 81%, respectively) did also not differ between the two outcome measures.

Furthermore, a strong, significant correlation was found between the total number of calls and the total duration of calls (Spearman's Rho = 0.83, Fig. 5).

3.5. Publication bias

Visual inspection of a funnel plot (Fig. 6) for the total number of vocalizations indicates that the plot is asymmetrical, due to an underrepresentation of imprecise studies reporting a neutral or negative effect. This finding indicates the possible presence of publication bias. These findings are in line with the results of Egger's test for small study effects, which indicates that plot asymmetry is present ($P < 0.0001$). Data were insufficient to perform a similar analysis for the total duration of calls.

Table 6
Subgroup statistics for total duration of vocalizations.

Subgroup	# Articles	# Experiments	# Animals	SMD[CI]
All studies	7	20	762	−1.70 [−2.14, −1.26]
Drug class				
Registered				
Test for subgroup differences NA				
SSRIs	5	5	181	−2.14 [−2.76, −1.51]
Anxiolytics/antidepressants miscellaneous	1	3	36	Not pooled
Experimental				
Test for subgroup differences NA				
NK_1 receptor antagonists	3	6	271	−1.52 [−2.08, −0.96]
Experimental miscellaneous	4	6	274	Not pooled
Origin				
Test for subgroup differences NA				
Colony	0	0	0	NA
Pregnant dam	3	10	250	−1.79 [−2.37, −1.21]
Dam with litter	2	9	402	−1.64 [−2.44, −0.83]
Not reported	1	1	110	−1.46 [−1.88, −1.03]
Age				
Test for subgroup differences NA				
Young < 17 days	1	6	72	−1.66 [−2.43, −0.89]
Mixed (4-31 days)	6	14	690	−1.70 [−2.22, −1.18]
Old > 18–26 days	0	0	0	NA
Test duration				
Test for subgroup differences NA				
5 min	5	12	664	−1.63 [−2.17, −1.09]
10–15 min	2	8	98	−1.84 [−2.61, −1.06]
> 30 min	0	0	0	NA
Selection				
Test for subgroup differences NS				
None	0	0	0	NA
Moderate/high vocalization	3	8	218	−1.67 [−2.31, −1.03]
High vocalization	4	12	544	−1.71 [−2.32, −1.11]
Repeated testing				
Test for subgroup differences NA				
Once	1	6	72	−1.66 [−2.43, −0.89]
2–3 times	2	4	440	−1.48 [−2.47, −0.49]
4 or more times	4	10	250	−1.79 [−2.37, −1.21]

NA not applicable; NS not significant; # number; SMD standardized mean difference; CI confidence interval; Subgroups consisting of less than three experiments and/or less than three articles were excluded from between subgroup analyses.

3.6. Sensitivity analysis

We performed a number of sensitivity analyses related to the classification and grouping of the experiments based on the drug properties. First, we excluded the experiment studying BMY7378 from the subgroup partial 5-HT_{1A} receptor agonists, since BMY7378, unlike buspirone, is not a registered, clinically active partial 5-HT_{1A} receptor agonist. Second, we excluded an experiment studying GR226,206, considered an inactive enantiomer, from the subgroup NK_1 receptor antagonists. Thirdly, we excluded an experiment studying S41744, a dual NK_1 receptor antagonist and serotonin re-uptake inhibitor from the NK_1 receptor antagonist subgroup. Exclusion of either GR226,206 or S41744 reduced heterogeneity in the NK_1 receptor antagonist subgroup for both the number and duration of calls. None of the sensitivity analyses had any significant effects on the direction or magnitude of the effect for either outcome measure.

4. Discussion

4.1. Pharmacological intervention

In this systematic review and meta-analysis, we studied the effect of registered anxiolytics and antidepressants with anxiolytic properties in the separation-induced vocalization test in guinea pig pups, examined potential differences between these classes, and assessed the effect of experimental drug classes in this test. We found that SSRIs, benzodiazepines and partial 5-HT_{1A} receptor agonists all reduced vocalization behaviour to a similar extent. Of the experimental compounds tested, NOP receptor agonists markedly reduced the number of vocalizations in guinea pig pups. The effect of this drug class was significantly stronger than that of SSRIs, benzodiazepines and partial 5-HT_{1A} receptor agonist.

Besides SSRIs and partial 5-HT_{1A} receptor agonists, several other serotonergic drug classes have been studied for putative anxiolytic and/or antidepressant actions. The 5-HT_{1A} receptor agonists flesinoxan and 8-OH-DPAT, the 5-HT_{1B} receptor antagonists AZD3783 and SB-616234-A, and the mixed 5-HT_{1D} agonist 5-CT have been tested in the guinea pig vocalization test (Dawson et al., 2006; Hudzik et al., 2003; Zhang et al., 2011). The findings in the current review do not support the suggestion that vocalization tests are particular sensitive to serotonergic compounds (Sanchez, 2003). Effects of these serotonergic compounds appeared low to moderate. 5-CT appeared to have the largest effect on vocalization behaviour. The mechanism of action of 5-CT is broad and includes agonist action at 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_2, 5-HT_3, 5-HT_{5A} and 5-HT_7 receptors (Yamada et al., 1998). Since no comparable compounds were tested, this large effect cannot readily be interpreted.

Meta-analysis showed that NOP receptor agonists strongly reduced the total number of vocalizations. Although all three

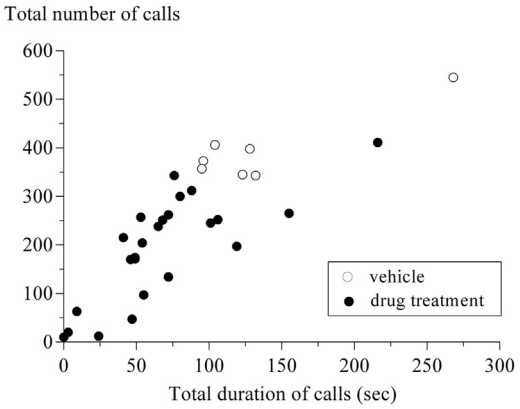

Fig. 5. Scatter plot of group mean values of total duration *versus* total number of calls obtained from dose-response studies reported in (Millan et al., 2010; Brocco et al., 2008; Chaki et al., 2005). Data points include both vehicle (open circles, $n=7$) and experimental conditions (closed circles, $n=24$).

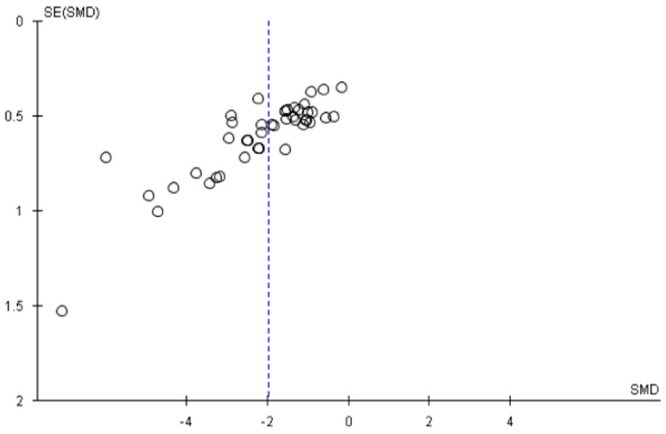

Fig. 6. Funnel plot for the outcome measure total number of vocalizations.

studies were performed in the same research laboratory, the fact that modest to low effects were reported for the benzodiazepine chlordiazepoxide, makes it unlikely that the large effects of NOP receptor agonists are solely due to specific testing conditions (Varty et al., 2005).

NOP receptors, also known as opioid like receptors, or nociception opioid receptors, are located throughout the human and rodent brain, including the cortex, extended amygdala, periaqueductal grey, raphe nuclei and hypothalamus (Mallimo and Kusnecov, 2013). The receptor shares 60% homology with opioid receptors, but neither the NOP receptor agonists tested, nor the endogenous ligand orphanin FQ/nociception [OFQ/N] bind to classic opioid receptors (Varty et al., 2005, 2008; Lu et al., 2011). Likewise, opioid receptor ligands do not bind to NOP receptors. Several disease states have been associated with changes in nociception levels, including anxiety and addiction, but potential therapeutic benefit of NOP receptor ligands has not yet been assessed in humans (Witkin et al., 2014).

Tachykinins, including substance P and neurokinin A, and NK_1 and NK_2 receptors are implicated in the regulation of stress related behaviour and the aetiology of anxio-depressive states (McCabe et al., 2009; Ebner et al., 2009; Millan et al., 2010; Harrison et al., 2001; Steinberg et al., 2002). In the isolation-induced vocalization test, three different drug classes targeting the neurokinin system were tested: NK_1 receptor antagonists, NK_2 receptor antagonists and substance P release inhibitors. Meta-analysis showed that all three drug classes reduced vocalization behaviour. Clinical trials of selective NK_1 antagonists on social anxiety and depression however, have yielded disappointing results (Millan, 2006; Poma et al., 2014)).

Both postsynaptic histamine H_1 and presynaptic H_3 receptors are thought to modulate emotion and stress regulation (Lamberty and Gower, 2004; Leurs et al., 2005). Although the histamine H_1 receptor antagonist, hydroxyzine is generally considered a weak anxiolytic, recent reviews concluded that hydroxyzine was equally effective to other anxiolytic agents, like benzodiazepines and buspirone (Huh et al., 2011; Guaiana et al., 2010). In the guinea pig pup vocalization test, hydroxyzine reduced total duration of calls, whereas the prototypic H_1 receptor antagonist chlorpheniramine was inactive. The authors pointed out that additional non-histaminergic effects are probably involved in the tranquilizing action of hydroxyzine (Lamberty and Gower, 2004). The two histamine H_3 receptor agonists tested, showed mixed results (Yokoyama et al., 2009; Lamberty and Gower, 2004). We could not further analyse these effects, since different outcome measures were used.

Morphine was one of the first compounds tested in the guinea pig pup vocalization test and proved rather effective in reducing the total number of calls (Herman and Panksepp, 1978), also relative to registered anxiolytics/antidepressants. Clearly, the actions of the opioid receptor agonist morphine are not comparable to those of standard anxiolytics. Importantly, though, the guinea pig pup vocalization test was originally studied in relation to development of social bonding and attachment (Herman and Panksepp, 1978; Nelson and Panksepp, 1998). The effects of morphine and the opioid receptor antagonist naloxone on vocalization behaviour in guinea pig pups indicate that endogenous endorphins also modulate emotional behaviour associated with social isolation and attachment (Herman and Panksepp, 1981; Nelson and Panksepp, 1998).

The modest reduction of isolation calls following treatment with the CRF_1 receptor antagonist CP154,526 (Hodgson et al., 2007), is interesting in light of the extensive work of Hennessy and co-workers regarding social isolation-induced behaviour in guinea pig pups. Guinea pig pups exhibit an initial active coping style, characterized primarily by vocalizing, which is then followed by a stage of passive behaviour in which pups display a crouched posture and extensive pilo-erection (Hennessy et al., 1995; McInturf and Hennessy, 1996). The work of Hennessy and co-workers indicates that subcutaneously administered CRF may accelerate the onset of passive behaviours, thereby reducing vocalization behaviour (Hennessy et al., 1991, 1997; Schiml-Webb et al., 2009). This is an important observation as it implies that a reduction in vocalization behaviour does not necessarily reflect a reduction in anxiety.

Apart from the above mentioned drugs, several other compounds have been tested: a bombesin BB_1/BB_2 receptor antagonist, a galanin GAL_3 antagonist, MCH_1 receptor antagonists, and a vasopressin V_{1B} receptor antagonist. These compounds all target neuropeptidergic systems that have been associated with emotional regulation (Holmes et al., 2003; Merali et al., 2006; Borowsky et al., 2002; Chaki et al., 2005; Swanson et al., 2005).These compounds were, however, tested only once and could not further be categorized for subgroup analysis. At global glance, most compounds had low to moderate effects on vocalization behaviour. It remains to be seen how these effects hold upon further testing, since publication bias may partly account for the positive effects.

4.1.1 Sensitivity and specificity of the separation-induced vocalization test in guinea pig pups Based on the current review, we conclude that the sensitivity of the separation-induced vocalization test in guinea pig pups for registered anxiolytics is high. All registered anxiolytics/antidepressants with anxiolytic properties were found active in the test. Almost all experimental compounds tested however, were also found active. This could be interpreted

in two ways. Due to publication bias, neutral and negative findings could be under represented. Alternatively, the translational value of the test may be moderate. Selective NK_1 receptor antagonists for instance, had an anxiolytic effect in this test, but proved disappointing in clinical studies (Millan, 2006). There were however, insufficient data to reliably determine pharmacological sensitivity and specificity of the test. Much to our surprise, even with regard to positive reference compounds, evidence was relatively scarce. Incidental studies, all from the same research laboratory, suggest that the test does have a certain level of specificity. The stimulant, d-amphetamine, and the antipsychotic haloperidol were rightly found inactive, as were the experimental anxiolytics ondansetron, a 5-HT_3 receptor antagonist, and CI-988, a CCK_2 receptor antagonist (Harro, 2006; Molewijk et al., 1996). On the other hand, the 5-HT_{1A} receptor agonist flesinoxan proved a false positive in the test (van Vliet et al., 1996).

Incidental studies further suggest that the test does not detect anxiogenic drug properties

(Molewijk et al., 1996), although this may well depend on the basal level of vocalization (see *e.g.* Groenink et al., 1996).

4.2. Methodological characteristics

The way in which the separation-induced vocalization test is performed, differs considerable between studies. Using subgroup analysis, we identified two test conditions associated with larger effect sizes upon pharmacological intervention: selection of animals with a moderate to high pre-test vocalization level and repeated testing of pups. Based on the data currently available, we conclude that origin and age of pups do not influence effect size.

Subgroup analysis showed that in studies that included all pups, irrespective of vocalization behaviour, smaller effects were found than those applying a selection criterion based on pre-test vocalization levels. Furthermore, with regard to effect size and precision, selection of pups with a high pre-test vocalization level (at least 120 s or 500 calls within 5 min) has no added value over selection of animals with a moderate to high vocalization level. Rupniak and co-workers (Rupniak et al., 2000) reported that over 50% of the pups did not qualify when using a criterion of over 300 s vocalization within 15 min). In case of selecting moderate to high vocalizers (>30 s within 5 min), approximately 7% of the pups was excluded from the experiment (information from authors (Brocco et al., 2008; Millan et al., 2010)). Thus, applying the moderate to high selection criterion could considerably reduce the number of animals discarded, without affecting effect size.

Subgroup analysis of the variable repeated testing showed that the effect of pharmacological treatment on the total number of calls was significantly smaller in pups tested once than in pups tested two or three times and pups tested more than three times. This finding suggest that repeated testing of pups is preferred for this test, since it appears to generate a larger effect size, and also reduces the total number of animals needed, relative to a between-subjects design, without reducing precision. Of note, in this analysis we did not take into account the additional reduction in variance generally obtained by applying a within subjects design, since none of the articles reported individual difference scores.

Although all commercially available guinea pig colonies were originally derived from one breeding colony (started by Dunkin and Hartley in 1926, personal communication S. Ripke, Harlan Laboratories, Venlo, Netherlands; see footnote), differences between colonies are likely to develop over time. Strain differences in anxiety behaviour and drug sensitivity are well documented for rats and mice (Van Bogaert et al., 2006; Griebel et al., 2000), but we are not aware of literature describing potential differences in anxiety behaviour between guinea pig strains. Surprisingly, none of the articles provided the official laboratory code of the strain used. Most articles did report the supplier, but suppliers may replace their colonies, in which case the generic name (*e.g.* Dunkin Hartley from Harlan UK) is still current, but the actual strain may well differ (see footnote). Therefore, it proved pointless to analyse strain differences in this test.[1]

In rats and mice, perinatal stress exposure can have considerably effects on anxiety behaviour (Sanchez et al., 2001). To the best of our knowledge, such behavioural effects have not been studied in guinea pigs. Available data indicate that test results obtained with pups transported shortly after birth, does not differ from that of pups obtained from transported pregnant dams. The data further suggest that if pups were bred in a laboratory colony (low perinatal stress), effect sizes were smaller, but this could not reliably be analysed.

Pettijohn (1979a) showed that vocalization behaviour remains relatively stable over the first four postnatal weeks, which is in line with our finding that age has no effect on treatment efficacy, although this finding could be confounded with the factor repeated testing. Though pups are still young when tested, sex differences could well influence vocalization behaviour. Guinea pigs can reach sexual maturity from four weeks of age (Trillmich et al., 2009), sex differences in brain morphology may occur before puberty (Severi et al., 2005), and prenatal stress may affect females differently than males (Schopper et al., 2012; Bauer et al., 2008). However, data were insufficient to assess the effect of sex on treatment efficacy.

Guinea pigs are communal animals, and vocalization among group members is important in their social interactions. The vocal repertoire of guinea pigs is highly developed: at least eleven different types of calls have been identified (Berryman, 1976), including whistles, purring and shrieking. The guinea pig pup separation calls have been classified as “whistles”, which are mainly audible sounds (Berryman, 1976), and probably serve as contact call for maternal attention (Pettijohn, 1977). Acoustic analysis comparing the first thirty calls with the last thirty calls within a 15 min isolation period, showed that whistle duration decreases, and mean frequency increases over time (Monticelli et al., 2004). Although initial studies specified the type of calls, it is unclear to what extent these or other calls are measured in the reported studies, since call characteristics are hardly reported.

In addition, the duration of a call may vary and change over time (Monticelli et al., 2004), and drugs may alter call characteristics, although this has hardly been studied (Wright et al., 2010). As the total time spent on vocalization is independent of individual call length, total duration of emitted calls may be a more reliable outcome measure than total number of calls. The current review however, indicates that with regard to sensitivity and precision, both outcome measures are equally valid to evaluate pharmacological interventions. Yet, since duration is a continuous measure, it is generally more suited for analysis of variance, the statistical method applied in most articles.

Test duration of a GPDV test is typically 5 min, although shorter (2 min) and longer tests have also been performed (from 12 to 15 min; Table 3). The effect of test duration could not be analysed, but the test periods used, are probably all within the optimal time window of separation calls. Hennessy and co-workers showed that vocalization behaviour markedly decreases and immobility levels increase after 30 min of social isolation (Hennessy et al., 1997, 1991; Hennessy and Moorman, 1989). In fact, studies using a considerable longer test duration (Table 3, 30 min or more), studied mechanisms

[1] Harlan UK (formerly known as OLAC) introduced the Dunkin Hartley strain in 1969 (laboratory code Ola:Dunkin Hartley), with animals derived from Porton. Harlan UK replaced the Ola:Dunkin Hartley by HsdPoc:DH (Dunkin Hartley, derived from Porcellus-animal-breeding-limited, Heathfield, UK) in the early nineties. In 2013, Harlan UK (and Harlan Netherlands) started breeding the HsdDhl:DH outbred strain (derived from David Hall and partners, UK). Charles River laboratories introduced the strain in 1968; nomenclature Hartley (Crl:HA), obtained from the Medical Research Council, UK.

underlying the shift from active to passive coping behaviour during prolonged social isolation (Hennessy et al., 2011a; Becker and Hennessy, 1993; McInturf and Hennessy, 1996).

Guinea pigs are stress sensitive animals and environmental stimuli may well affect their vocalization behaviour and sensitivity to drugs. Presence of the mother in the test cage prevented the actions of a CRF receptor antagonist on vocalization behaviour (Hennessy et al., 1997; Pettijohn, 1979a), whereas presence of the experimenter in the test room reduced overall vocalizations (personal communication PM Verdouw). Likewise, testing in a dimly lit room, in a closed cabinet, reduces the vocalization response (Hennessy and Ritchey, 1987; Arch-Tirado et al., 2000), as does repeatedly disrupting the litter within a short time window (Arch-Tirado et al., 2000; Hennessy et al., 2006). Presently, reporting of these experimental details is poor and should therefore be greatly improved.

4.3. Reporting of measures to reduce risk of bias and biases in publication

The quality of included articles was low, and visual inspection of the funnel plot indicated that neutral and negative studies are probably underrepresented in our data set.

It is possible that most authors merely failed to report study characteristics and measures to reduce bias, which are fundamental to good scientific practice, but there is reason for concern. Several recent systematic reviews on experimental animal studies have shown that methodological rigor is low in many fields of preclinical research, which may lead to inflated treatment effects (Sena et al., 2014). Together with the frequently observed risk of publication bias (Sena et al., 2010; see also below) and the difficulties in obtaining missing data from the authors, valuable information is lost to the scientific community. This may result in misconception of *e.g.* disease mechanisms and drug efficacy, as well as redundant animal studies and unnecessary duplications. Although there is no guarantee that the translational value of animal models will increase with improved conduct and reporting of preclinical research, we strongly support the implementation of publication guidelines such as the ARRIVE guidelines and the gold standard publication checklist (GSPC), to optimize the information provided in publications and to improve awareness regarding study quality (Kilkenny et al., 2010; Hooijmans et al., 2011).

4.4. Strengths and limitations

We systematically and objectively quantified the effect of pharmacological interventions in the guinea pig pup vocalization test. We identified methodological factors that may improve test sensitivity and reduce the number of animal needed.

It proved impossible to study the effect of individual drugs. Categorizing drugs into classes may conceal particular characteristics of a drug, relative to other drugs within its class. If indicated, we performed sensitivity analysis to control for this. Also, no data were available to actively control for potential muscular or sedative effects, which may occur at higher doses and could interfere with vocalization behaviour.

Finally, overall study quality was poor, which may have caused an overestimation of drug efficacy in the individual studies, as well as in our meta-analysis.

4.5. Concluding remarks and recommendations

The separation-induced vocalization test in guinea pig seems suited as global screen to detect potential anxiolytic drug properties. There were however, insufficient data to reliably determine pharmacological sensitivity and specificity of the test. Selecting pups with moderate to high levels of vocalization and repeated testing of pups are associated with larger treatment effects. Reporting of study characteristics was poor, risk of bias high and there is possible publication bias present in this field of research. We therefore urgently call for proper conduct and reporting of experimental animals studies in this field, as well as publication of all available data. These conditions are crucial to further our understanding of the translational value of animal models for anxiety disorders and the pharmacological treatment thereof.

Conflict of interest

The authors declare that they have no conflicts of interest. In the past five years, LG received research grants/support from Utrecht University (focal area Neuroscience & Cognition), Grünenthal GmBh, Aachen, Germany, Institut de Recherches Servier, Croissy-sur-Seine, France, and Psychogenics Inc., New York, USA.

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