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Abstract

Despite widespread reports that intranasal application of oxytocin has an exuberant variety of behavioural effects, very little of the huge amounts applied intranasally appears to reach the CSF. However, peripheral concentrations are raised to supraphysiological levels, with likely effects on diverse targets including the gastrointestinal tract, heart and reproductive tract. The wish to believe in the effectiveness of intranasal oxytocin appears to be widespread, and needs to be guarded against with scepticism and rigor. Pre-registering trials, declaring primary and secondary outcomes in advance, specifying the statistical methods to be applied, and making all data openly available should minimise problems of publication bias and questionable post hoc analyses. Effects of intranasal oxytocin also need proper dose-response studies, and need to include controls for peripheral effects, by administering oxytocin peripherally and by blocking peripheral actions with antagonists. Reports in the literature of oxytocin measurements include many that have been made with discredited methodology. Claims that peripheral measurements of oxytocin reflect central release are questionable at best.

Introduction

More than 100 neuropeptides are expressed in different neuronal subpopulations. Whereas neurotransmitters are packaged in abundant small vesicles targeted to nerve endings, peptides are packaged in large vesicles that are relatively sparse, and which can be released from all compartments of a neuron. These vesicles carry a large cargo (~85,000 molecules of oxytocin) and peptides act at receptors with nanomolar affinity (1). Often, receptors are densely expressed at sites innervated by few fibres that contain the peptide ligand, indicating that neuropeptides are more like hormones than neurotransmitters, acting at sites distant from their point of release, with organisational and activational roles rather than roles in information processing *per se* (2).

Some neuropeptides have a startling ability to evoke particular behaviours. Central injections of oxytocin trigger satiety and enhance sexual behaviour in animal models; in rats and sheep they can trigger maternal behaviour (3,4), in monogamous voles they facilitate pair bonding (5), and oxytocin-receptor deficient mice show disturbances in social behaviour.

Recently, there has been a deluge of reports that oxytocin affects social behavior in humans when delivered as a nasal spray, and in some studies when delivered peripherally (6). Such effects have several possible explanations. Oxytocin might enter the CNS, mimicking

"neurohormonal" oxytocin release (2), or might act peripherally to indirectly affect behaviour, either via oxytocin receptors or vasopressin receptors activated at high concentrations of oxytocin. Other possibilities are that reported effects reflect methodological weaknesses, and post-hoc interpretation of outcomes with minimal statistical rigor.

Oxytocin and the blood-brain barrier

Most of the body's oxytocin is stored in the posterior pituitary, which, in the adult rat, contains 0.5-1μg oxytocin and similar amounts of vasopressin. This gland contains the nerve endings of magnocellular neurons whose cell bodies lie in the hypothalamus, but it lies outside the blood-brain barrier, so peptide released from these endings readily enters the blood. The rat pituitary contains enough vasopressin to maintain the normal plasma concentration of 1pg/ml for 30 days, and a concentration of 10pg/ml, as seen during water deprivation, for three days (1).

Between the blood and interstitial fluid of the body there is no barrier to the passage of peptides, so the distribution volume for oxytocin is much larger than the plasma volume (7). Oxytocin is stable in plasma (except in pregnancy, when oxytocinase is abundant), and is cleared from the blood via the kidneys and liver. In the rat, at i.v. doses of up to 500ng/kg, oxytocin disappears from the blood with a half-life of 3-8min (8). The half-life in CSF is longer: 28min in guinea pig (9), and 19min in rat (10). Oxytocin is thought to be cleared from CSF by a combination of flow into the subarachnoid space (11), and active transport into blood (12).

In man, the pituitary oxytocin content (estimated by bioassay) is ~14IU (28μg) (13). Circulating concentrations are (as in the rat) ~1-10pg/ml, and the pharmacokinetics after i.v. injection fit a two-compartment model, with a distribution volume of ~33L, a distribution half-life of ~3min and an elimination half-life of ~20min (14). As in the rat, ~1% of oxytocin is excreted in urine (15).

After entering the blood, oxytocin rapidly penetrates extravascular fluid, but does not cross the blood-brain barrier in appreciable amounts. In an early study, Ermisch *et al*. gave rats intracarotid injections of radiolabelled oxytocin: brain areas without an effective blood-brain barrier extracted up to 30-fold more peptide than other brain regions, but oxytocin failed to penetrate deeper into the brain (16). Brain areas that lack a blood-brain barrier are encapsulated by glial and endothelial cells that form tight junctions, preventing passage of peptides both to deeper brain regions and from them.

The effectiveness of the blood-brain barrier for oxytocin was measured by Mens *et al*., who injected 5µg subcutaneously in rats, increasing plasma concentrations 500-fold to ~38,600pg/ml (10). Increases in CSF were modest; concentrations increased from ~40pg/ml to ~150pg/ml. The authors calculated that just 0.002% of the injected oxytocin had reached the CNS after 10min, when CSF concentrations were maximal.

Oxytocin penetration of the brain after intranasal administration

Two routes have been proposed for the passage of peptides from nose to brain. The first postulates internalization of peptide into olfactory or trigeminal neurons, followed by axonal transport and exocytosis. There is doubt about whether peptides survive internalisation, and Born *et al*. dismissed this as requiring hours for substances to reach the brain by axonal transport (17). Oxytocin might pass through intercellular clefts into the subarachnoid space, but transport across the arachnoid membrane is not an important route for the entry of solutes into brain (18). The arachnoid is a multi-layered epithelium with tight junctions between cells of the inner layer that form an effective seal; valve-like villi project into the sagittal sinus through the dura and only allow CSF movement from the brain to blood. However, if vast amounts of peptide accumulate in the subarachnoid space, the concentration difference across the blood-brain barrier might support non-specific passage. The slow disappearance of oxytocin from blood after intranasal application suggests that large amounts reach an extravascular pool from which it slowly leaches into the circulation.

Ang and Jenkins studied the brain penetration of radiolabelled vasopressin given i.v., and, importantly, measured how much label was still associated with intact peptide (19). Vasopressin, like oxytocin, is a nonapeptide with a sulphur bridge, differing in just two amino acids, and has similar bioavailability. Plasma vasopressin disappeared with the expected bi-exponential decay, while CSF levels of the label were maximal after 50min; this peak was <1% of that in plasma, and none of the label in CSF was associated with intact peptide. They also gave labelled vasopressin intranasally, sampling CSF and plasma 40min later. The concentration of label in CSF was ~5% of that in plasma, but whereas 16.5% of the label in plasma was associated with intact peptide, *none* of the label recovered from CSF was.

Since then, six studies have measured CSF levels of oxytocin or vasopressin following intranasal application. Born *et al.* reported that, after giving 40IU (80µg) in man, CSF levels rose within 10min from ~5pg/ml to ~10pg/ml, increasing to ~20pg/ml at 60min (17). They administered, as a bolus, more than twice the pituitary vasopressin content, justifying this dose on the basis that most probably passes through the nose without being

absorbed. Estimating the CSF volume as 300ml, it seems that ~4.5ng of vasopressin reached the CSF: 0.005% of the given dose – assuming that the rise was due to administered peptide and not endogenous release triggered indirectly.

Striepens *et al*.(20) measured CSF oxytocin in 11 patients given 24IU oxytocin intranasally. Whereas Born *et al*. saw an increase after 10min (albeit with vasopressin, at a larger dose) (17), Striepens *et al.* saw no increase at 45 or 60min. However, three patients sampled at 75min had CSF levels 64% higher than controls (at ~30pg/ml). In the same month, the authors submitted a paper on fMRI changes in subjects tested 30min after intranasal oxytocin (21). That paper does not cite the CSF data, or the fact that the fMRI measurements were made at times when CSF oxytocin was unchanged.

Neumann *et al*. gave 20µg of oxytocin intranasally to rats (20 times the pituitary content) and found no change in CSF oxytocin after 45min (22). However, they found a doubling of oxytocin levels in microdialysates of brain regions collected at 30-60min, correlated with a four-fold rise in plasma. Intracranial microdialysis inevitably ruptures blood vessels around the probe, so these measurements might reflect local passage into the brain from damaged vessels.

Dal Monte *et al*. gave 48IU oxytocin (~10µg/kg body weight) intranasally to macaques, using either a spray or nebuliser (23). These increased CSF oxytocin from ~35pg/ml to ~90pg/ml after 40min. On the (very) conservative assumption that the CSF/ECF volume in the macaque is 40ml, then the additional content at this time is 2.2ng, 0.002% of the administered dose.

Chang *et al*. gave 25IU oxytocin to two macaques, and reported a rise in CSF from ~20 to ~50pg/ml at 35min (24). In a larger study, Modi *et al*. gave 24IU oxytocin (~5µg/kg body weight) to macaques by spray or aerosol; only the aerosol produced a significant increase in CSF (from ~20 to ~60pg/ml) (25). Again assuming a CSF volume of 40ml, the additional content at this time is 1.6ng - 0.003% of the administered dose. Both spray and aerosol raised plasma oxytocin levels. Intravenous administration of the same dose raised plasma levels to ~60,000pg/ml with no increase in CSF.

All seven studies administered enormous amounts of peptide intranasally – in every case more than the pituitary content as a bolus – yet found only modest rises in CSF: two found no rise. At most, 0.005% of intranasally injected oxytocin reaches the CSF within an hour. Intranasal application achieves higher concentrations of peptide in blood than in CSF, and, as basal concentrations in plasma are lower than in CSF, the proportional change in blood is much greater.

How much oxytocin must enter the brain for a behavioral effect?

Although intranasal application seems inefficient, doses of oxytocin that have become conventional in human studies all exceed the pituitary content of oxytocin. Thus, given that enormous amounts are given, the tiny rate of penetration might still allow biologically relevant amounts of peptide to enter the brain. If 24IU oxytocin were delivered as a bolus intravenously, the peak plasma concentration would exceed 1,400pg/ml, three orders of magnitude higher than physiological concentrations.

Are these enormous intranasal doses enough to deliver effective concentrations of oxytocin into the brain? In lactating rats, suckling evokes bursts of action potentials in oxytocin cells that result in pulsatile oxytocin secretion, and this bursting is facilitated by 1-2ng oxytocin i.c.v. (26). Effects on maternal behavior in the rat need much higher doses (~400ng) (4). Partner preference effects in voles require infusions of oxytocin at 10-100ng/h (5), and, to stimulate maternal behavior in sheep, it seems necessary to deliver 5µg i.c.v. (3).

It is unsurprising that higher concentrations of oxytocin are needed for behavioral effects than for peripheral effects. As in most G protein-coupled receptors, agonist stimulation of oxytocin receptors leads to desensitization (27). Receptors at peripheral sites are exposed to much lower concentrations of oxytocin (1-10pg/ml), than receptors in the brain (1), so will be more sensitive to it.

Thus 1ng oxytocin is the lowest i.c.v. dose shown to elicit a behavioural effect in animal studies, and 2ng induces expression of the immediate-early gene c-*fos* in rat brain regions where oxytocin receptors are expressed, including in the amygdala and hypothalamus. By contrast, intranasal application of 1µg oxytocin in rats produced no activation at these or any other sites in the forebrain (28) - and no activation in the olfactory bulb, the postulated primary target of interneuronal transfer of oxytocin. Maejima *et al.* reported that intranasal administration of a higher dose of oxytocin (10µg) activated Fos expression at the paraventricular nucleus, the area postrema and the dorsal motor nucleus of the vagus (29). The area postrema is outside the blood-brain barrier, and should not be accessible to oxytocin from the CSF. These same areas were also activated by oxytocin given systemically.

Peripheral consequences of intranasal oxytocin

Although intranasal applications deliver only modest rises in CSF concentrations, they produce large and prolonged increases in circulating oxytocin, to levels far above those

needed for physiological effects. Oxytocin receptors are widely distributed in the periphery: their presence on mammary tissue and uterus is well known, but there are many other sites of expression (30). Fifty years ago, intranasal oxytocin was commonly used to augment labor, using doses much lower than used lately, despite the high levels of pregnancy oxytocinase that must be overcome for oxytocin to exert a uterotonic effect. Hoover studied 1,806 women who had been given intranasal oxytocin during childbirth (31). Labor was stimulated by 1-4 doses of 0.4-0.8IU given at 20-min intervals - a total of, at most, 3.2IU. Equivalent effects were achieved by intravenous infusion of 1-2mU/min, giving rise to the estimate that ~1% of intranasally-applied oxytocin enters the circulation (32).

Intranasal application of oxytocin or vasopressin, at doses currently used, delivers supraphysiological concentrations into the circulation. Born *et al*. achieved plasma concentrations of 20pg/ml after giving 40IU vasopressin (17). These are higher than Robertson *et al*. reported for any patient, including those with pathologically elevated vasopressin secretion; in man, maximal urine concentrating ability is achieved at a vasopressin concentration of ~5pg/ml (33). Of the above-mentioned studies, four (20,22,24, 25) achieved oxytocin concentrations in excess of 20pg/ml from basal levels of <5pg/ml, while Dal Monte *et al*. reported a rise to 80pg/ml after nasal spray, but no significant rise with a nebulariser (23). Modi *et al*. reported a 100-fold increase in plasma in the macaque (to >300pg/ml), and a rise to ~60pg/ml (from 10pg/ml) after nebulariser application (25).

Peripheral targets for oxytocin

Oxytocin regulates feeding and metabolism at multiple sites (34,35). Its receptors are expressed throughout the gastrointestinal tract, and on gastric vagal nerve endings (36). Intranasal application in dogs increases glucagon and insulin secretion (37), and this is probably mediated peripherally as intravenous oxytocin has a similar effect in goats (38) and dogs (39). In rats, oxytocin receptors are expressed by glucagon- and insulin-secreting cells in the pancreas (40), and direct stimulation of glucagon release has been characterised *in vitro* (41). Oxytocin also affects gastric motility: it is secreted in response to food intake, and slows gastric emptying (42).

At the ventromedial nucleus of the hypothalamus, oxytocin promotes both satiety and sexual receptivity – enhancing the lordosis effect in female rats (43); this nucleus contains virtually no oxytocin fibres so is a likely target of dendritic secretion from magnocellular oxytocin neurons (35). Some oxytocin cells of the paraventricular nucleus project to the spinal cord, where they regulate penile erection (44). However, oxytocin is also released from

the pituitary during sexual arousal, and, in the male reproductive tract, oxytocin acts on the vas deferens to facilitate sperm transport (45), in the prostate gland (46) to promote ejaculation, and on the penis (47) to promote erection.

Oxytocin receptors are expressed in the heart, coupled to secretion of natriuretic hormone (48). Oxytocin has direct cardiac effects, and intranasal application in man increases heart rate variability (49). At the anterior pituitary, oxytocin is a releasing factor for prolactin, and has effects on other endocrine cells too (50). In man, intravenous oxytocin has been reported to inhibit ACTH release (51), although in animal models oxytocin seems to have a predominantly stimulatory effect (52). Measurements of plasma corticosterone after intranasal oxytocin have reported mixed effects; one recent study reports a rise in stress-evoked secretion (53). Oxytocin receptors are also present on bone (54) and in the thymus (55). Finally, oxytocin at moderately high concentrations is an agonist at V1 vasopressin receptors, and these are expressed at many peripheral sites (including the olfactory epithelium (56); the consequences of activating these is not known). These peripheral actions of oxytocin seem likely to have some behavioural consequences – especially those on reproductive organs, the heart and gastrointestinal tract.

However, it is not impossible that enormous amounts of oxytocin delivered intranasally achieve biologically significant elevations in the brain. Oxytocin is avidly degraded in brain tissue, as known from the fact that CSF concentrations of oxytocin-associated neurophysin are much higher than those of oxytocin. This neurophysin, a fraction of the peptide precursor, is secreted in equimolar amounts to oxytocin, but is not enzymatically degraded in brain. Comparing CSF levels of neurophysin and oxytocin suggests that only ~5% of the oxytocin that is released in the rat brain reaches the CSF (1). Thus, intranasally applied oxytocin *might* penetrate some brain regions yet not enter the CSF. However, it seems inappropriate to cite CSF measurements as if they demonstrate that substantial brain penetration occurs when they show minimal penetration at best, and it is disconcerting that the high levels of oxytocin achieved in the periphery are assumed to have no behavioral consequences. Effects of intranasal oxytocin need proper dose-response studies, and need to include controls for peripheral effects, by administering oxytocin peripherally and by blocking peripheral actions with antagonists.

Measuring oxytocin and vasopressin

Many studies have drawn conclusions from highly questionable measurements of plasma oxytocin, and others mistakenly claim that plasma measurements reliably reflect

oxytocin release in the brain. Validated radioimmunoassays have long converged on the conclusion that basal circulating levels of oxytocin and vasopressin in man are in the range 1-10pg/ml, confirmed recently by combined LC/mass spectrometry (57). However, assays of unextracted plasma mainly measure immunoreactivity that is chemically and physiologically unrelated to vasopressin or oxytocin, and mainly contained in high molecular weight fractions. Robertson *et al*. showed that two radioimmunoassays for vasopressin yielded measurements in unextracted plasma that were at least two orders of magnitude greater than those inferred from other evidence, and which did not fluctuate in parallel with endogenous authentic vasopressin (33,58). Eliminating high molecular weight elements by extraction subsequently became standard in labs measuring oxytocin or vasopressin in plasma.

However, many papers have used an ELISA on unextracted plasma, yielding values of >300pg/ml (59-65). In response to caustic criticism (66), the manufacturers "strongly recommended" that plasma samples should be extracted to avoid matrix interference (67), a recommendation reinforced by Christensen et al. (68), but this advice is still being ignored. Any hope that the measured levels correlate with authentic oxytocin levels seems vain. Three studies have compared ELISA oxytocin measurements of the same samples with and without extraction (69-71): all reported no correlations.

One of those papers concludes that adolescent exposure to oxytocin increases plasma oxytocin in adulthood, and it might be expected that this conclusion was drawn from data on extracted plasma (70). Not so: the data from extracted samples showed no differences between groups: instead, the authors built their interpretation on the measurements of unextracted plasma.

The discrepancy between measurements in unextracted and extracted plasma is two orders of magnitude, but when Wismer Fries *et al*. reported that urinary excretion of oxytocin and vasopressin in orphans was affected by early neglect, they reported levels that were, for both peptides, nearly a million fold too high, after applying a method that was neither sensitive enough or selective enough to measure either peptide in urine (72), a report that attracted pointed criticism (73,74). The authors have since improved their methodology, and in subsequent studies report values in line with classically validated measurements (75).

Central and peripheral release of oxytocin

The lack of access to central measures of oxytocin has led some to turn to peripheral measures of oxytocin in the belief that these are convergent. Central oxytocin derives from at least three separate systems. Some magnocellular neurons project sparsely to some other brain areas, including the amygdala and septum, but it seems likely that most of the central innervations derives from non-neuroendocrine neurons of the paraventricular nucleus (76) that do not project to the pituitary. For example, oxytocin is released from neurons that project to the caudal brainstem, regulating gastric reflexes (77), and from neurons that project to the spinal cord which are involved in penile erection (44).

Oxytocin *is* released into the brain in large amounts from the soma and dendrites of neurons that project to the pituitary– but this is semi-independent of axonal release, being governed in part by mobilisation of intracellular calcium, a mechanism not present at the terminals (2). In response to α-melanocyte stimulating hormone (acting at MC4 receptors), oxytocin is released from dendrites of magnocellular neurons, but their electrical activity and peripheral oxytocin secretion is inhibited (78). In response to i.v. cholecystokinin, oxytocin is released into the blood and in the hypothalamus (79), but several other agents affect release differentially: for instance, in dogs, opioids stimulate peripheral secretion but suppress central secretion (80). Hyperosmotic stimuli increase oxytocin release from both dendrites and nerve terminals in rats, but on different time scales; dendritic release is increased as plasma concentrations fall (2).

Appetite-related stimuli and some reproductive stimuli activate both central and peripheral oxytocin release, but the timings and extent of these actions differ, and differences are exaggerated by the different pharmacokinetics in the two compartments. Oxytocin is released into blood and brain during parturition, but in sheep (81), plasma concentrations were only elevated for 15min postpartum whereas those in CSF were increased for >120min.

Stress affects both central and peripheral secretion of oxytocin– but while swim stress in rats increases oxytocin release in the hypothalamus and into plasma (82), novelty stress increases CSF but not plasma concentrations (83). In lactating rats, oxytocin is released in the hypothalamus in response to suckling *before* any peripheral secretion (84), but in guinea pigs, simultaneous measurements revealed a large increase in plasma during suckling but no change in CSF (85), leading the authors to conclude that CSF levels reflect secretion from centrally projecting neurons that are functionally independent of the magnocellular neurosecretory neurons.

In lactating rhesus monkeys, Amico *et al.* (86) found that "variations in the concentrations of oxytocin in CSF were independent of the suckling stimulus and plasma

oxytocin concentrations" and noted, as others had before (87), that CSF levels but not plasma levels show a circadian variation. They concluded that "release of oxytocin into the CSF of lactating monkeys is disassociated from release into the peripheral circulation" (86).

Winslow *et al.* measured CSF and plasma oxytocin in rhesus monkeys in a study of the effects of rearing conditions: while CSF oxytocin correlated with social behaviour, plasma levels did not, nor did they correlate with CSF levels collected in the same session (88). CSF and plasma concentrations showed no correlation in patients with aneurysmal subarachnoid haemorrhage (89), or in either suicide attempters or healthy volunteers (90), or in non-neurological and nonpsychiatric patients under basal conditions (91), or in MDMA users (92). Carson *et al*. reported that CSF and plasma oxytocin concentrations are correlated in children, but only after correcting the data for multiple variables that led to independent release (93). Oxytocin is released into the blood at orgasm in men (94), but Kruger *et al.* found no changes in CSF at any stage of the sexual response cycle (95).

Publication bias

Much of the interest in intranasal oxytocin followed a report that it enhanced trust (96); extravagant data interpretations and unorthodox uses of statistics in some of these studies have been incisively criticised (97), and such defects appear to be widespread. The unreliability of small clinical trials is recognised, and attributed to a combination of publication bias, questionable statistical analysis and methodological weaknesses, and there are similar concerns about basic biological research (98). Ferguson and Heene argued that for psychological research " the field often constructs arguments to block the publication and interpretation of null results and that null results may be extinguished through questionable researcher practices", resulting in the promulgation of theories that are "ideologically popular but have little basis in fact" (99). A survey of researchers in psychology suggested that practices such as excluding outliers *post-hoc*, using multiple outcome measures and only reporting results that reached statistical significance, and halting data collection to test for significance and resuming if significance is not found are common (100). Simmons *et al*. warned of practices that transform null findings into positive findings by statistical adjustments or the exercise of undisclosed "researcher degrees of freedom" (101), they showed, by simulations and experiments, how easy it is to accumulate "statistically significant" evidence for a false hypothesis.

Pre-registering trials, declaring primary outcomes in advance, specifying statistical methods to be applied, and making data openly available should minimise these problems.

Several recent trials conform to some of these conditions, particularly in reporting clear primary outcomes. They show no effect of intranasal oxytocin on patients with schizophrenia or healthy volunteers (102,103); or in early psychosis (104); or on individuals with Prader-Willi syndrome (105); or in MDMA users (92); or in youths with autism spectrum disorders (106,107). Revealingly, in the last study "caregivers who believed their children received oxytocin reported greater improvements than caregivers who believed their child received placebo." The wish to believe in the effectiveness of intranasal oxytocin appears widespread, and needs to be guarded against.

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